Effects of Heroin on Sickness Behavior and Proinflammatory Mediators in Associated Brain Regions

Alison Fay Wagner

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology (Behavioral Neuroscience).

Chapel Hill

2010

Approved by:

Advisor: Donald T. Lysle, Ph.D.

Reader: Regina M Carelli, Ph.D.

Reader: Mark Hollins, Ph.D.

Reader: Mitchell J. Picker, Ph.D.

Reader: Todd E. Thiele, Ph.D.

Abstract

ALISON FAY WAGNER: Effects of Heroin on Sickness Behavior and Proinflammatory Mediators in Associated Brain Regions

(Under the direction of Donald T. Lysle, Ph.D.)

Proinflammatory mediators in the brain are associated with a constellation of adaptive behaviors known as sickness behaviors. Although many studies have confirmed peripheral immunosuppressive effects of opiates, none have demonstrated effects of heroin or any other opiate on immune responses within the central nervous system. The following experiments examined the effects of heroin on brain proinflammatory mediators alone and during an immune challenge, and the effects that heroin has on sickness behaviors and proinflammatory mediators in associated brain regions.

Experiments in Chapter 2 established that heroin produces a reliable, short-term hyperthermia that is not reversed by pre-treatment of indomethacin, nor does it correlate with increased proinflammatory mediators in the hypothalamus, the area of the brain most associated with temperature changes. However, we did show that in the presence of an immune challenge (lipopolysaccharide, LPS), heroin appeared to have a suppressive effect on some proinflammatory mediators 90 minutes after treatment.

In Chapter 3, we further investigated these findings and examined both LPS-induced fever response and increased proinflammatory mediators in the hypothalamus, and determined that heroin has a suppressive effect on all proinflammatory mediators measured. In addition to the proinflammatory mediator suppression, heroin also attenuated fever production induced by LPS.

In Chapter 4, we examined the effects of heroin on LPS-induced proinflammatory mediators in the hippocampus, a region associated with behavioral depression. As in the hypothalamus, the hippocampus exhibited suppressed proinflammatory mediators when heroin treatment occurred concurrently with LPS treatment, although the hippocampus selectively showed these effects in interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Additionally, heroin treatment diminished behavioral depression induced by LPS.

Taken together, these studies indicated that although heroin produced hyperthermia, heroin treatment does not acutely elevate proinflammatory mediators in the brain (Chapter 2), and when it is given in the presence of LPS, heroin has an immunosuppressive effect on both production of proinflammatory mediators and sickness behaviors (Chapters 3 and 4). Overall, these experiments demonstrate that heroin suppresses immune responses in the brain that are normally key parts of the process of producing adaptive sickness behaviors.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
CANX	calnexin
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
COX-2	cyclooxygenase-2
δ	delta
DLAM	Division of Laboratory Animal Medicine
DRN	dorsal raphe nuclei
GIRK	G protein-coupled inwardly-rectifying potassium
Gp120	glycoprotein 120
IACUC	Institutional Animal Care and Use Committee
IκBα	inhibitory factor kappa B alpha
IL-1β	interleukin-1beta

IL-6	interleukin-6
IL-10	interleukin-10
iNOS	inducible nitric oxide synthase
icv	intracerebroventricular
κ	kappa
kg	kilogram
LC	locus coeruleus
LTP	long-term potentiation
LPS	lipopolysaccharide
μ	mu
μl	microliter
μg	microgram
MCP-1	monocyte chemotactic protein-1
mRNA	messenger ribonucleic acid
mg	milligram
MRN	median raphe nuclei
NFκB	nuclear factor kappa B

NIH	National Institutes of Health
NTS	nucleus of the solitary tract
RNA	ribonucleic acid
PAG	periaqueductal gray
PGE_2	prostaglandin E 2
POA	preoptic area
s.c.	subcutaneous
SEM	standard error of mean
TNF-α	tumor necrosis factor-alpha

Chapter 1

General Introduction

Overview

It has become increasingly apparent that immune cells and their products in the central nervous system (CNS) are essential to a number of immunologically relevant behaviors. Opiates have been shown to cause immunosuppression in the periphery; however, no studies have addressed the effects of opiates on central immune responses. The experiments described herein are the first to examine the effects of opiates on immune activation in the CNS.

Brief overview of the immune system

The immune system is divided into two major components: adaptive and innate. The adaptive immune system includes T and B cell responses, and requires a lengthy response time to form specialized responses to microbial infections. The innate immune system is more relevant to this dissertation and performs less specialized but more immediate responses to microbial infections. This part of the immune system plays an important role by performing as the first line of host defense against microbial infection (Iwasaki & Medzhitov, 2010). Within the innate

immune system, there are many types of cells, and of these, macrophages will be most discussed here. In addition to phagocytizing (or consuming) foreign cells, macrophages are responsible for the release of protein messengers known as cytokines. When host defense mechanisms are engaged (such as during a bacterial or viral attack), macrophages secrete proinflammatory cytokines, which aid the adaptive immune system and help to destroy the foreign invaders. These proinflammatory responses are vital to an effective host defense. Proinflammatory cytokines released from resident macrophages and astrocytes, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and monocyte chemotactic protein-1(MCP-1), are integrally involved in assisting important inflammatory responses, including the production of nitric oxide via inducible nitric oxide synthase (iNOS) (Hart, 1988b; Cannon, 2000; Thompson, Karpus, & Van Eldik, 2008). Throughout this document, the proinflammatory cytokines listed above and other proinflammatory non-cytokines will be collectively referred to as proinflammatory mediators. The proinflammatory mediators function as an essential first line response to microbial infection in addition to recruiting other immune cells to the site of infection (Wang et al 2008).

Proinflammatory responses in the central nervous system

In the CNS, cells are generally either neurons or glial cells. Often in neurobiology, the focus is almost exclusively on neurons. However, glial cells outnumber neurons 10:1 in the brain, and these cells are becoming increasingly

popular topics of study due to new findings that glial cells have vital roles in regulation of neuronal communication. Within the glial cells, microglia are often targets for studies examining modulation of neuron health and neural communication. The responses of microglia have become an important area of study due to their important effects on neurons, so in order to understand these cells, some understanding of the functioning of the immune system is necessary.

As a subset of macrophages, microglia in the brain are also responsible a number of roles, including sensing pathological events in the CNS, supporting neurons, and releasing proinflammatory mediators (Kreutzberg, 1996; Perry, Hume, & Gordon, 1985). In addition to serving the immune functions above, the proinflammatory mediators perform important signaling functions to affect neuronal communications. While inflammatory responses by the innate immune system are well characterized in the periphery, less is known about the importance of these responses within the CNS. It has only become apparent in the past 15-20 years that these immune responses exist in the brain at all; prior to this time, the brain was believed to be “immune privileged” and not subject to the normal immune response due to the blood brain barrier. In fact, it is now abundantly clear that proinflammatory cytokines are can be induced in the brain during immune system activation, particularly in the hypothalamus and hippocampus (Laye, Parnet, Goujon, & Dantzer, 1994; O'Connor et al., 2003).

A common method for studying the immune response *in vivo* is by using lipopolysaccharide (LPS), a Gram negative bacterial cell wall fragment which is

recognized by innate receptors, resulting in an immune response similar to that seen in an active infection. This allows for experiments examining the immune response without the complicating factor of a live infection. In laboratory settings, stimulation by LPS produces a proinflammatory effect with elevated TNF-α, IL-1β, and IL-6 in the brain, particularly in the hypothalamus and hippocampus (Chen et al., 2008; Richwine, Sparkman, Dilger, Buchanan, & Johnson, 2009). Many of the proinflammatory mediators induced by LPS, including IL-1β, IL-6, TNF-α, iNOS, MCP-1, are all regulated by a common transcription factor, nuclear factor kappa B (NFκB) (O'Neill & Kaltschmidt, 1997). Activated NFκB is responsible for the proinflammatory effects of LPS in microglia (Nakajima, Matsushita, Tohyama, Kohsaka, & Kurihara, 2006). It is possible that this is one mechanism that can be manipulated to alter proinflammatory mediator production.

Sickness behaviors

Communication between the immune system and the central nervous system by cytokines is now known to be integral to a constellation of behaviors, collectively known as sickness behaviors. Sickness behaviors, rather than being a side effect of immune activation as previously thought, are believed to be a directed and organized behavioral response to aid the organism in recovering from infection (Hart, 1988a; Maier & Watkins, 1998b). The brain regions where the most cytokine activity is seen include the hippocampus and hypothalamus, and these regions are the ones most associated with sickness behaviors. Cytokines were first proposed to

have an active role in the brain by Dantzer and Kelley (Dantzer & Kelley, 1989). Since that time, a number of cytokines have been definitively linked to sickness behaviors, particularly fever production. These cytokines include TNF-α, IL-1β, and IL-6 (Conti, Tabarean, Andrei, & Bartfai, 2004; Kluger, Kozak, Leon, Soszynski, & Conn, 1995).

Sickness behaviors consist of a number of beneficial immune responses, including production of fever, a reduction in social activities and general activity, fatigue or sleepiness, and anorexia (Dantzer & Kelley, 2007; Hopkins, 2007; Campisi et al., 2003; Conti et al., 2004; Hori et al., 1991; Conti et al., 2004; Kluger et al., 1995; Blatteis, Li, Li, Feleder, & Perlik, 2005; Jansky et al., 1995; Konsman, Parnet, & Dantzer, 2002; Dantzer et al., 2007). These behaviors are exhibited during infection and have evolved as adaptive responses to increase survival during bacterial or viral attacks (Kluger, Kozak, Conn, Leon, & Soszynski, 1996; Kluger, Kozak, Conn, Leon, & Soszynski, 1998). Sickness behaviors are now believed to represent a shift in motivation state to reorganize the organism's resources towards fending off infection (Dantzer et al., 2007). These behaviors are adaptive in a number of ways, most centering on the production of fever.

Fever is a complex but essential aspect of sickness behavior and a vital component of the immune system response to infection. The idea that fever is an adaptive immune process was believed as long ago as ancient Greece, and this idea has reemerged with substantial scientific evidence behind it (Mackowiak, 1998). Fever production is an evolutionarily conserved adaptation that can be seen in

animals ranging from humans to mollusks, which indicates the significance this behavior (Kluger, 1978). Fever has a number of advantageous responses, including causing a more efficient functioning of leukocytes and macrophages (Sebag, Reed, & Williams, Jr., 1977; Manzella & Roberts, Jr., 1979). These changes translate to effects on disease outcome, as recent studies have shown that the suppression of a normal fever response during infection increases morbidity for many diseases, and allowing fever to be produced is beneficial in most cases (Kluger et al., 1998; Kluger et al., 1996).

The production of fever has been studied extensively for many decades, which allows for a solid foundation of knowledge of mechanisms. Although there is some debate about alternative pathways, the classical "fever pathway" is initiated by the production of endogenous pyrogens, which are typically proinflammatory cytokines. In particular, IL-1β and IL-6 are associated with the playing a pivotal role in the onset of fever, although TNF-α and other proinflammatory mediators have also been shown to have pyrogenic activity (Kluger et al., 1995; Dinarello, 2004; Harden, du, I, Poole, & Laburn, 2006; Harden, du, I, Poole, & Laburn, 2008; Kozak et al., 1995; Kozak et al., 1998; Stefferl, Hopkins, Rothwell, & Luheshi, 1996). These pyrogenic factors then trigger production of prostaglandin E_2 (PGE_2) in the hypothalamus (Nilsberth, Hamzic, Norell, & Blomqvist, 2009b; Nilsberth et al., 2009a; Griffin, 1999). The synthesis of PGE_2 via the inducible enzyme cyclooxygenase-2 (COX-2) is traditionally viewed as the final and obligatory step in the classical fever pathway, directly affecting thermosensitive neurons in the hypothalamus which then regulate physiological responses to produce an increase

in body temperature (Nilsberth et al., 2009b). See Figure 1 (page 11) for a schematic of the traditional fever pathway.

While fever is associated with the hypothalamus, behavioral depression as noted by reduction of activity is associated with the hippocampus, a more recent development in this field (Konsman et al., 2008; Harden et al., 2006; Harden et al., 2008; Konsman et al., 2002; Linthorst, Flachskamm, Holsboer, & Reul, 1994). A reduction of activity is not immediately apparent as evolutionarily adaptive until one considers the costliness in terms of energy of fever. While fever is clearly adaptive for mounting an effective immune attack in order to clear microbes from the body, it is metabolically expensive, and a reduction in general activity helps to compensate for this cost and allows the redirection of energies towards producing fever- this is the change in motivational state mentioned above. High levels of proinflammatory mediators such as TNF-α and IL-6 in the hippocampus have been implicated in increased release of hippocampal serotonin, which then results in behavioral depression (Pauli, Linthorst, & Reul, 1998; Anisman, Gibb, & Hayley, 2008; Linthorst & Reul, 1998). For the purposes of this dissertation, behavioral depression will refer to the reduction of overall activity, although some researchers include decreased sociability and reduction of feeding/drinking behaviors in this classification.

Opiate-induced immunosuppression

Opiates such as morphine and heroin have been linked to producing immunosuppression through a number of mechanisms, as well as having a variety of effects on body temperature. The consequence of this is seen in opiate abusers who suffer from an increased rate of a variety of bacterial infections. Opiates are known to produce immunosuppression in the peripheral system (including spleen and blood) through central nervous system pathways, particularly by suppressing activation of the innate immune system and proinflammatory cytokines such as IL-1β and TNF-α (Wang, Barke, Ma, Charboneau, & Roy, 2008; Chuang et al., 2005; Mellon & Bayer, 1998). However, essentially nothing is known regarding the effect of opiates on immune responses that occur *within* the central nervous system. One study indicated that morphine decreases fever response, which was correlated with decreased serum proinflammatory mediators (Mayfield, Kozak, Rudolph, & Kluger, 1998). Given the accepted importance of CNS proinflammatory mediators in producing fever, the impairment of fever by opiates as found by Mayfield et al. suggests a suppression of proinflammatory mediators in the CNS. However, no published reports have directly examined the effects of morphine or heroin on proinflammatory mediator levels in the brain. Due to the established peripheral immunosuppressive effects of these opiates, it is likely that opiates also produce immunosuppression in the CNS. Impairments of proinflammatory mediators in the CNS, given the role of these molecules in sickness behavior, may also translate to alterations of adaptive sickness behaviors normally mediated by proinflammatory mediators in the CNS.

Interaction of opiates and body temperature

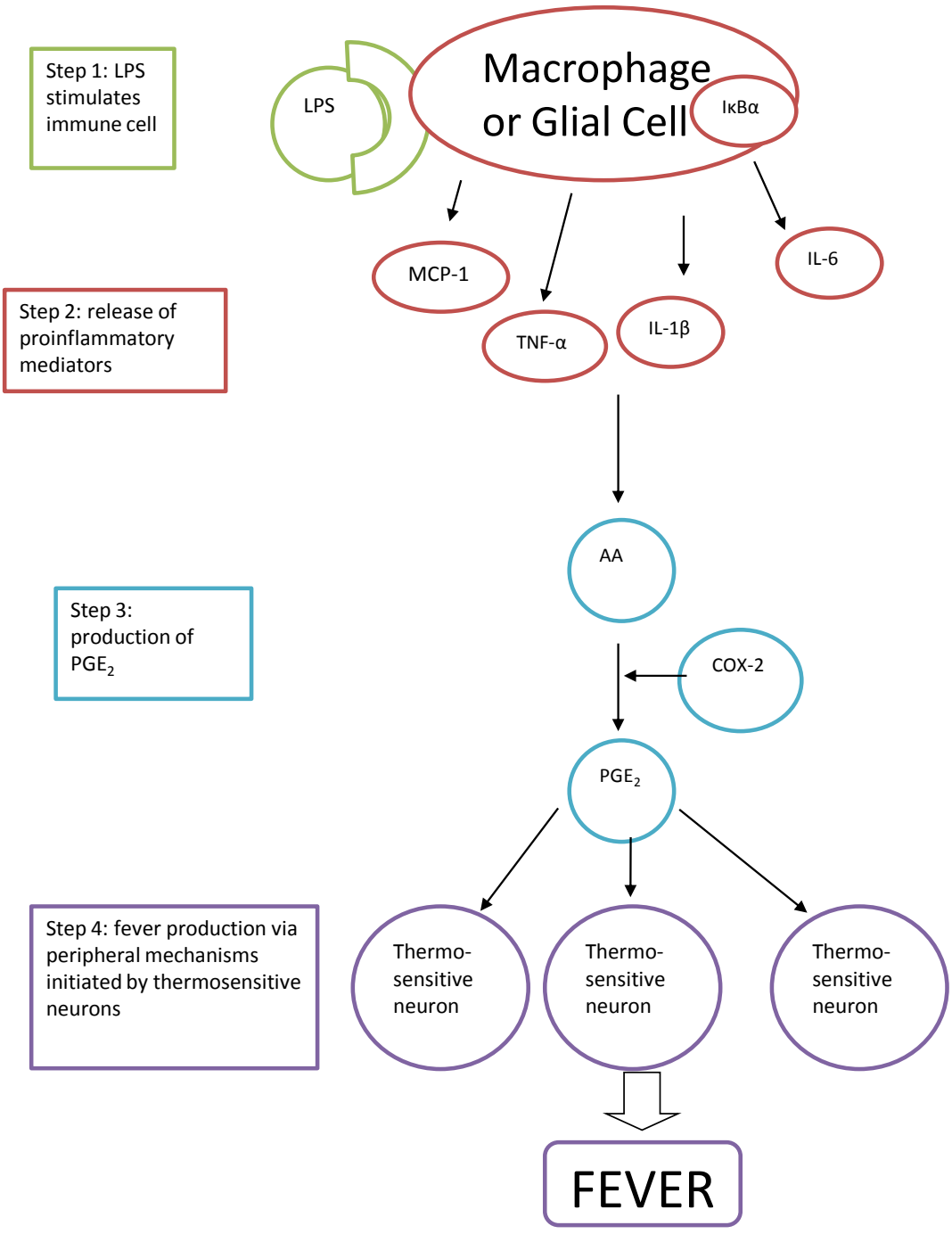
In addition to inhibiting LPS-induced fever, morphine and other μ opioid receptor agonists have independent effects on body temperature. Low to moderate doses of morphine induce hyperthermia through actions on the μ opioid receptors, although high doses can produce hypothermia through actions on the κ or δ opioid receptors (Baker & Meert, 2002; Geller, Hawk, Tallarida, & Adler, 1982). However, this pathway may be distinct from that of the classical fever pathway due to conflicting evidence of the necessity of PGE_2 as well as a more rapid onset of effects (1-2 hours) that subsides much more quickly compared to traditional fever (Wallenstein, 1983; Prakash & Dey, 1981). The end result of hyperthermia due to MOR activation is, like fever, mediated by thermosensitive neurons in the hypothalamus, but the pathway leading to this outcome is not fully understood (Yakimova, Sann, & Pierau, 1996). The interaction between exogenous opiates such as heroin and morphine through μ receptors in thermal regulation by both in impairing fever and inducing hyperthermia has yet to be addressed comprehensively. Interestingly, morphine treatment has also been shown to downregulate proopiomelanocortin (POMC), the precursor protein to the endogenous opioid β-endorphin, which also binds to the μ receptor (Mocchetti, Ritter, & Costa, 1989). Heroin addicts also have impaired β-endorphin responses in thermal regulation, indicating that endogenous opioids may also play a role in thermoregulation and exogenous opiates may alter these responses (Vescovi & Coiro, 1993; Vescovi et al., 1989). The seemingly contradictory results -- opiate-

induced hyperthermia yet opiates suppress LPS-induced fever – have yet to be comprehensively addressed.

Overview of experiments

The experiments described in the following chapters evaluated the effects of opiates on body temperature and activity levels as well as how these behaviors correlated with stimulation of proinflammatory mediators. These experiments take on a number of problems that have not been previously addressed in the literature. The overarching goal of these aims is to evaluate the effects of heroin on behavioral depression and fever as immunologically relevant behaviors and proinflammatory mediators in associated brain regions. Chapter 2 evaluated the effects of heroin on core body temperature and proinflammatory mediators during heroin-induced changes in the brain, specifically the hypothalamus. Chapter 3 furthered the investigations by examining fever production by LPS and expression of proinflammatory mediators in the hypothalamus with and without concurrent heroin administration. Chapter 4 measured proinflammatory mediator expression in the hippocampus induced by systemic LPS treatment and determined if co-administration of heroin can inhibit the expression of proinflammatory mediators in the hippocampus, as well as evaluated the behavioral depression seen under these conditions as measured by general activity.

Figure 1. Schematic of traditional fever pathway



Chapter 2

Heroin-induced hyperthermia is not regulated by proinflammatory mediators associated with the traditional fever pathway.

Introduction

Opiates have complex effects on the regulation of body temperature. Morphine has a dose-dependent effect on body temperature, with high doses causing hypothermia and low doses causing hyperthermia (Dafters & Taggart, 1992; Geller, Hawk, Keinath, Tallarida, & Adler, 1983). It has become clear that the hyperthermic effects of morphine are mediated through the μ receptor, as these are prevented by μ antagonists as well as antisense oligonucleotides designed to block the μ receptor, while the hypothermic effects are mediated by the κ receptors (Thornhill, Hirst, & Gowdey, 1978; Chen, Geller, DeRiel, Liu-Chen, & Adler, 1996; Bhargava, Rahmani, Villar, & Larsen, 1993; Handler, Geller, & Adler, 1992). Self administered heroin also produces a hyperthermia that can be reversed with naloxone and is not associated with activity increases, indicating that the rise in body temperature is centrally mediated (Kiyatkin & Wise, 2002). These thermic effects occur very quickly after opiate treatment, even when given systemically, usually between 30 minutes and 3 hours. These effects are more rapid than the typical

fever in response to an immune challenge, but the involvement of proinflammatory cytokines is unknown. As discussed before and elsewhere in this dissertation, fever involves an increase in proinflammatory cytokines and PGE_2 to affect thermosensitive neurons in the hypothalamus. Evidence indicates that morphine and heroin can produce a brief burst of proinflammatory cytokines within the first hour of treatment, followed by a period of depressed cytokine production (Pacifici, Di, Bacosi, Pichini, & Zuccaro, 2000). However, it is unknown if this brief increase in proinflammatory cytokines occurs in the brain or if that phenomenon relates to the thermic effects of opiates.

The effects of opiates on body temperature are even more complex when an immune challenge is present. Given the abundant evidence that μ opiates typically suppress proinflammatory mediators in the periphery, it perhaps should not be surprising that morphine suppresses fever production by LPS, which was correlated with decreased plasma levels of IL-6 and TNF-α (Mayfield, Kozak, Rudolph, & Kluger, 1998). The suppression of fever suggests that opiates have suppressive effects on proinflammatory cytokines in the brain, specifically in the hypothalamus; however, no studies to date have examined the relationship between opiates, proinflammatory cytokines in the hypothalamus, and fever. If heroin temporarily increases proinflammatory mediators as the study by Pacifici suggests, the short-term increase in proinlammatory mediators could affect the ability of the hypothalamus to respond appropriately to an immune challenge. The following studies were performed to determine if the hyperthermic effect of heroin is mediated by proinflammatory cytokines and PGE_2, similar to fever.

Overview of experimental procedures.

In order to examine the hyperthermic responses induced by heroin, a number of experiments were performed.

Experiment 1a. Based on pilot studies, we sought to examine a low (1mg/kg) and a high (10mg/kg) dose of heroin in reference to effects on body temperature. Rats (n=24) were given 0mg/kg, 1mg/kg, or 10mg/kg of heroin while body temperatures were measured.

Experiment 1b. In this experiment, we aimed to determine if pretreatment with indomethacin (10mg/kg), an inhibitor of prostaglandin synthesis, would prevent the hyperthermic response of heroin (1mg/kg). Indomethacin was dissolved in DMSO and rats (n=8) were administered either vehicle or indomethacin. One hour later, all rats were given 1mg/kg of heroin.

Experiment 1c. In this experiment, rats (n=24) were given 1mg/kg of heroin or saline and 1mg/kg of LPS or saline. LPS was included in this study to determine if the cytokine response was altered in the presence of an immune challenge during the hyperthermic response by heroin. Rats were then sacrificed at 90 minutes post-treatment, which previous studies have shown is during the peak hyperthermic response to heroin. Brain tissue was dissected and processed immediately for mRNA analysis of proinflammatory mediators via RT-PCR. Target molecules include the proinflammatory cytokines TNF-α, IL-1β, IL-6, and MCP-1, as well as

iNOS, IκBα as a measure of NFκB activation, and COX-2 as a measure of PGE_2 synthesis activity. IκBα is a protein that must be removed and degraded to allow for the translocation of NFκB into the nucleus (for review of NFκB, see O'Neill & Kaltschmidt 1997). The production of new IκBα is indicative of degradation of IκBα that occurs during NFκB activation, and levels of IκBα mRNA are used as a measure of NFκB activation (Zhao et al., 2007).

Materials and Methods

Animals

All methods were approved by University of North Carolina at Chapel Hill's Institutional Animal Care and Use Committee (IACUC) and are in accordance with National Institutes of Health (NIH) guidelines as an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. All animals were individually housed on a 12:12 light:dark cycle in temperature-controlled Division of Laboratory Animal Medicine (DLAM)-approved facility with lights on at 7:00AM. All experimental manipulations took place during the dark portion of the light cycle. Rats were allowed at least one week to habituate to the new environment prior to any experimental manipulations. Food and water were available *ad libitum*. For all experiments, Lewis male rats were purchased at approximately 2-3 months in age (Charles River Laboratories, Raleigh, NC). There were a total of 48 rats used for these experiments.

Surgical procedures

Surgeries were performed to implant biotelemetry devices within the abdominal cavities of the rats. The rats were anesthetized by administering a 0.3 ml intraperitoneal injection of ketamine (90 mg/ml) and xylazine (10 mg/ml). A small incision was made down the midline of the abdomen through the dermis, followed by an incision to the linea alba. The E-Mitter (Mini-Mitter, Sunriver, OR) was placed in the abdominal cavity and sutured to the abdominal musculature. The abdominal musculature and the dermis were sutured separately. The rats were allowed at least one week to recover before further experimental procedures were conducted.

Home cages were placed directly on top of the biotelemetry receivers, which then transmitted data recordings of core body temperature and activity measures to VitalView software on a computer located within the room (Mini-Mitter, Sunriver, OR). Body temperatures were recorded at one minute intervals for the duration of the experiments.

Drug administration

All injections of heroin, indomethacin, LPS, or saline were given subcutaneously on the abdomen. LPS was E. coli derived and purchased from Sigma-Aldrich (St. Louis, MO). Indomethacin was also purchased from Sigma-Aldrich.

Dissection procedures

Immediately following cervical dislocation, brains were extracted rapidly and target regions (hypothalamus, hippocampus, cortex, striatum) were dissected immediately. Target tissues were dissected using Paxinos and Watson as a guide (Paxinos & Watson, 2005), extracting the entire hypothalamus, entire hippocampus, cortical tissue immediately dorsal to the hippocampus, and striatum tissue immediately ventral to the hippocampus. Tissues were stored in RNAlater (Ambion, Austin, TX) at -80°C until processed for RNA extraction.

Real time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from each of these brain tissues using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH), following modified methods described by Chomczynski & Sacchi (1987). The RNA was quantitated spectrophotometrically (GeneQuant II, Pharmacia-Biotech, Piscataway, NJ). Reverse transcription was performed using Oligo(dt)18 primer and Moloney Murine Leukemia Virus-Reverse transcriptase following the protocol of the Advantage™ RT-for-PCR Kit from Clontech (Palo Alto, CA). PCR amplications were performed using the LightCycler TaqMan Master kit (Roche Diagnostics, Indianapolis, IN), in which reaction components were prepared in a master mix solution. Each reaction consisted of 17µl of master mix with 3µl of sample placed in glass capillary tubes designed for the LightCycler system. Capillaries were then placed in the LightCycler

instrument (Roche Diagnostics). The samples and master mix were pre-incubated for 10 minutes at 95 degrees Celsius and amplified for 45 cycles of 10 seconds at 95 degrees followed by 30 seconds at 60 degrees using specifically designed PCR primer sets for each target molecule (TNF-α, IL-1β, iNOS, MCP-1, IL-6, IkBα and COX-2) that were synthesized by the Nucleic Acids Core Facility (Lineberger Cancer Center, University of North Carolina at Chapel Hill) (see Table 1 for sequences). Fluorescence level was determined at the end of the amplification phase for each cycle of PCR. Each run included negatives and an internal standard curve.

Data and Statistical Analysis

Body temperature data analysis. Body temperature data was analyzed based upon samples made at every half hour point that was averaged over three data points (e.g., for measurement of half an hour after treatment, data from the 29, 30, and 31 minutes post-treatment were analyzed and represented the 30 minute data point for each rat). Body temperatures were followed for 120 minutes after heroin treatment.

Proinflammatory mediator mRNA data analysis. To ensure comparable quality of RNA among samples, the housekeeping gene calnexin (CANX) was assessed and target molecule data were expressed as a ratio to amount of CANX measured.

Statistics. All statistics were performed using SPSS v17.0. For Experiment 1a, repeated measures ANOVA was used to determine if there was a time * heroin interaction. For Experiment 1b, a repeated measures ANOVA was used to

determine if there was a time * indomethacin interaction. If interactions were found, Tukey posthoc tests were performed. For mRNA data from Experiment 1c, ANOVAs were performed examining the main effects and interactions for LPS and heroin for all proinflammatory mediators, and Tukey posthoc tests were also performed when significant effects were found. Significance was set at p=0.05.

Results

Experiment 1a

Pilot studies and published experiments suggested that 1mg/kg and 10mg/kg of heroin have very different effects on body temperatures. Experiment 1a furthered that exploration of body temperature effects by heroin and investigated high (10mg/kg) and low (1mg/kg) doses. The repeated measures ANOVA revealed a significant time * heroin interaction [$F(1,64)=3.278$, $p \leq 0.01$]. Posthoc tests indicated the 1mg/kg heroin treated group was significantly elevated compared to the saline treated group treated group post-treatment at 30 minutes ($p \leq 0.01$), 60 minutes ($p \leq 0.05$), and 90 minutes ($p \leq 0.05$). The 10mg/kg treated group did not differ from either group at any time point as a result of a large amount of variability, which was also seen in the pilot studies conducted in our laboratory as a result of this large dose. From these results, we conclude that 1mg/kg of heroin reliably induces a hyperthermic effect, and thus can be used for future studies evaluating the effects of heroin **(Figure 2.1a).**

Experiment 1b

This experiment evaluated whether pre-treatment with indomethacin, a prostaglandin synthesis inhibitor, would prevent the hyperthermic effect of heroin. A repeated measures ANOVA based on the 2 hours of data post-treatment with heroin (and three hours post-indomethacin pretreatment) indicated that there was no difference between indomethacin pre-treated rats and those treated with vehicle [$F(4,24)=0.329$, $p=0.856$]. The lack of difference between the two groups indicates that indomethacin did not affect the hyperthermic response by heroin, and prostaglandins are likely not necessary for production of this response. There was, however, a significant effect of time [$F(4,24)=12.424$, $p\leq0.001$], and paired sample t-tests confirmed that body temperature was elevated (compared to baseline) at 60 minutes [$t(7)=5.960$, $p\leq0.001$], 90 minutes [$t(7)=7.432$, $p\leq0.001$], and 120 minutes [$t(7)=6.499$, $p\leq0.001$] post-treatment with heroin, thus confirming that heroin in both groups increased body temperature at these time points. There were no differences in body temperatures prior to these time points, indicating that neither indomethacin nor the vehicle had any immediate effects on body temperature **(Figure 2.1b)**.

Experiment 1c: RT-PCR

Hypothalamus. The primary purpose of this experiment was to examine hypothalamic proinflammatory mediator mRNA during the hyperthermic peak

produced by heroin; however, we also decided to examine other brain regions for comparison. In the hypothalamus, there was a main effect of LPS in TNF-α [$F(1,22)=10.803$, $p\leq0.01$], IL-1β [$F(1,22)=18.410$, $p\leq0.001$], MCP-1 [$F(1,22)=7.818$, $p\leq0.01$], IL-6 [$F(1,22)=7.678$, $p\leq0.01$], and IκβΑ [$F(1,23)=8.955$, $p\leq0.01$] mRNA. In addition to the main effect of LPS, IL-6 mRNA also demonstrated a main effect of heroin [$F(1,22)=7.761$, $p\leq0.01$] and an interaction of LPS * Heroin [$F(1,22)=4.255$, $p\leq0.05$]. Posthoc tests revealed that LPS/saline treatment increased IL-1β mRNA compared to saline/saline and heroin/saline treated groups, but there were no differences between the saline/LPS and heroin/LPS groups. IκBα mRNA levels were significantly different only between the heroin/saline treated groups and the saline/LPS treated groups, while IL-6 mRNA levels indicated that the saline/LPS treated group was significantly elevated compared to all three other groups. **(Figure 2.2)**

Hippocampus. A 2 (LPS) by 2 (heroin) ANOVA for each proinflammatory mediator measured was performed. There were no significant results from the hippocampus. **(Figure 2.3)**

Cortex. In the cortex, there were no significant effects of LPS, heroin, or interactions of the two in the measures of any of the proinflammatory mediators measured, with the exception of IL-6, which had a significant main effect of LPS [$F(1,20)=6.256$, $p\leq0.05$]. Posthoc tests revealed only a difference between the heroin/saline treated group and the LPS/saline treated group ($p\leq0.05$), but importantly, there were no differences compared to the saline/saline treated group. **(Figure 2.4)**

Striatum. A 2 (LPS) by 2 (heroin) ANOVA for each proinflammatory mediator measured was performed. There was an interactive effect of LPS * heroin in the measure of TNF-α mRNA [$F(1,19)=6.621$, $p \leq 0.05$]; posthoc tests revealed only a difference between the heroin/LPS treated group and saline/LPS treated group, indicating that the heroin/LPS treated group had significantly higher levels of TNF-α mRNA compared to the saline/LPS treated group. IL-1β mRNA analysis demonstrated a significant main effect of LPS [$F(1,19)=4.943$, $p \leq 0.05$]; however, none of the posthoc tests were significant. Likewise, MCP-1 mRNA showed a main effect of LPS [$F(1,20)=4.342$, $p \leq 0.05$], but no groups were revealed to be significantly different than the others. These results indicate that LPS has a slight effect in elevating proinflammatory mediators, but it is not a robust effect, and it is not affected or is suppressed by heroin co-administration in the striatum. **(Figure 2.5)**

Discussion

Major findings

These experiments demonstrated clearly that 1mg/kg heroin induces a hyperthermic effect that is not reversed by PGE_2 and does not rely on the same mechanisms as traditional fever; that is, the hyperthermic effect of heroin does not induce proinflammatory mediators or depend on synthesis of PGE_2 in the hypothalalmus during increased body temperature to produce this effect.

Proinflammatory mediator mRNA during peak of heroin-induced hyperthermia

The first two experiments in this chapter indicated that heroin induces a hyperthermic effect which peaks at 90 minutes post-treatment. We investigated whether opiates such as heroin cause this fast hyperthermic response through a relatively quick increase in proinflammatory cytokines. The results indicate that heroin treated rats, with or without LPS, did not show increased proinflammatory mediator mRNA, and in fact, the only group that showed any increased responses was the LPS/saline treated group. These results clearly indicate that heroin does not increase proinflammatory cytokine production during the peak of hyperthermia, and this phenomenon is distinct from fever production due to an immune challenge (such as LPS, as in these experiments). We must consider, then, other possibilities for the thermic response of opiates.

Direct effects of opiates on hypothalamic neurons

Within the hypothalamus, there are three distinct types of neurons that are relevant to fever production: warm sensitive neurons, temperature insensitive neurons, and cold sensitive neurons. Warm sensitive neurons comprise about 30% of the neurons in this region, and when activated, induce behaviors that cool the body (i.e., these neurons sense warmth and respond accordingly to sustain homeostasis). Cold sensitive neurons only make up about 5% of the POA, and the

effects of these neurons are the opposite of the warm sensitive neurons, although their small number renders them less studied than the others (Griffin, 1999). Alterations in these neurons produce a change in temperature set point, which can result in hyperthermia (in this case, fever) or hypothermia. Inhibition of warm sensitive neurons results in a body temperature set point change upwards, or hyperthermia; indeed, IL-1β and presumably other proinflammatory cytokines decrease activity of warm sensitive neurons in the hypothalamus in a PGE_2-dependent manner (Nakashima, Hori, Mori, Kuriyama, & Mizuno, 1989; Griffin, 1999; Boulant, 1998).

Interestingly, morphine has been reported to have direct but conflicting effects on the activity of warm sensitive neurons in the POA, with some studies reporting a decrease in activation while others reported an increase in neuron firing (Baldino, Jr., Beckman, & Adler, 1980; Yakimova et al., 1996). In the Baldino et al experiment, morphine caused an increased firing of warm sensitive neurons, which would presumably lower body temperature in a whole organism, and this effect was antagonized by naloxone. In the Yakimova et al experiment, the investigators demonstrated that morphine decreased sensitivity of warm sensitive neurons, which can be extrapolated to mean that morphine would cause an increase in body temperature. This effect was reversed by the selective μ antagonist CTOP. The difference in the studies is minute, but it is possible that naloxone, while selective for μ, also had an antagonistic action at other opioid receptors, namely κ receptors. Since κ agonists increase excitation in warm sensitive neurons from the POAH, an antagonism of these receptors would account for the results seen by Baldino et al

(Yakimova, Sann, & Pierau, 1998). An excitation of κ receptors by morphine has been shown to occur and moreover, to induce a hypothermic effect. Therefore, it seems more likely that activation of μ receptors can induce a hyperthermic effect by directly acting on hypothalamic thermosensitive neurons.

Our results indicating that heroin does not increase proinflammatory cytokines in the hypothalamus during the hyperthermic peak indicates that cytokine activity is not likely to play a role in in the thermic effects of heroin, and it is more likely that opioid receptors themselves are responsible for these effects.

Conclusion

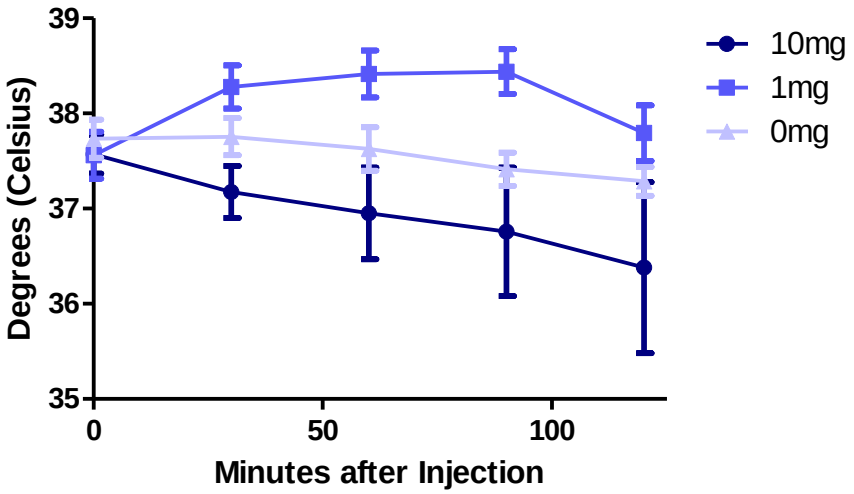
It can be concluded that the hyperthermic effect induced by heroin does not rely on the traditional fever pathway to produce the rise in body temperature, as evidenced by the lack of increased proinflammatory mediators in the hypothalamus or alteration by a PGE_2 synthesis inhibitor. However, the question of how heroin affects fever, and the potential for disruption in the production of proinflammatory mediators as suggested in the results of IL-6 mRNA in this chapter has yet to be investigated. The following studies examine potential mechanisms through which heroin might disrupt fever production.

Table 1. Primer information used for RT-PCR.

Gene	5' Primer	5' Size	3' Primer	3' Size	Amp Size
CANX	agcaagcctaaagcagagga	20	gaccatatttgcaggggaag	20	133
TNFα	gggcctccagaactccag	18	gagcccatttgggaacttct	20	139
IL-1β	agcttcaggaaggcagtgtc	20	tcccacgagtcacagagga	19	149
iNOS	tgaggattacttcttccagctca	23	tgggtgtcagagtcttgtgc	20	130
MCP-1	cagaaaccagccaactctca	20	gtggggcattaactgcatct	20	144
IL-6	cctggagtttgtgaagaacaact	23	ggaagttggggtaggaagga	20	142
IκBα	ccaactacaacggccaca	18	ctgtccggccattacagg	18	123
COX-2	gaccaggagtacactttcaaacag	24	attccttcccccagcaac	18	123

Figure 2.1 **(a)** Heroin dose-dependently affects core body temperature. Body temperature was measured via 1 minute samples taken over 3 minutes and averaged per rat at every half hour interval. Body temperature measurements indicate that a dose of 1mg/kg (s.c.) of heroin increased body temperature compared to treatment with saline, while a dose of 10mg/kg (s.c.) of heroin did not produce any significant effects due to variability. **(b)** Heroin (1mg/kg, s.c.) produced an increase in core body temperature compared to baseline core body temperature. Heroin-induced hyperthermia was not affected by indomethacin treatment (10mg/kg, s.c.). Data is represented as means by treatment group with standard error of means (SEM). * denotes significance of at least $p \leq 0.05$.

a



b

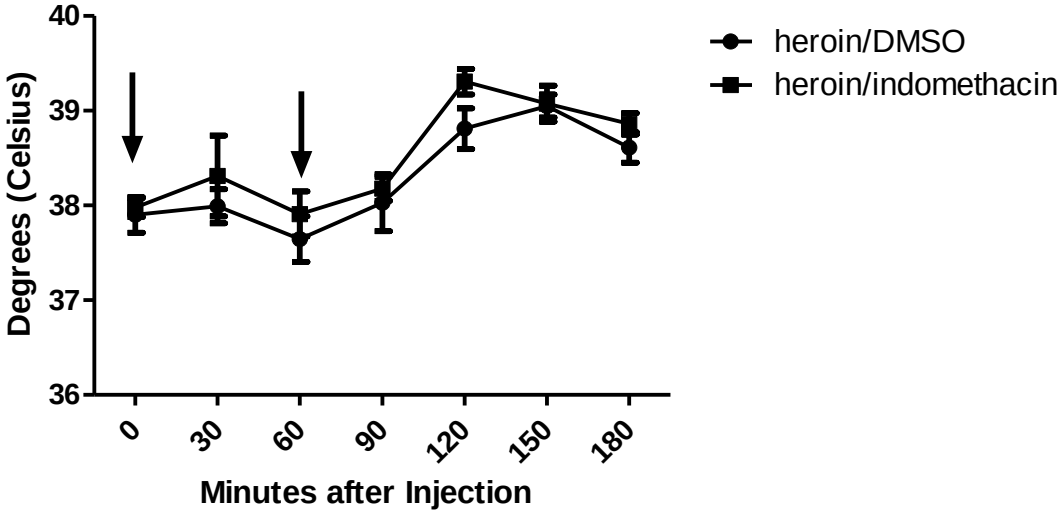


Figure 2.2 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 90 minutes after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels measured in the hypothalamus. **(a)** TNF-α **(b)** IL-1β **(c)** iNOS **(d)** IL-6 **(e)** MCP-1.

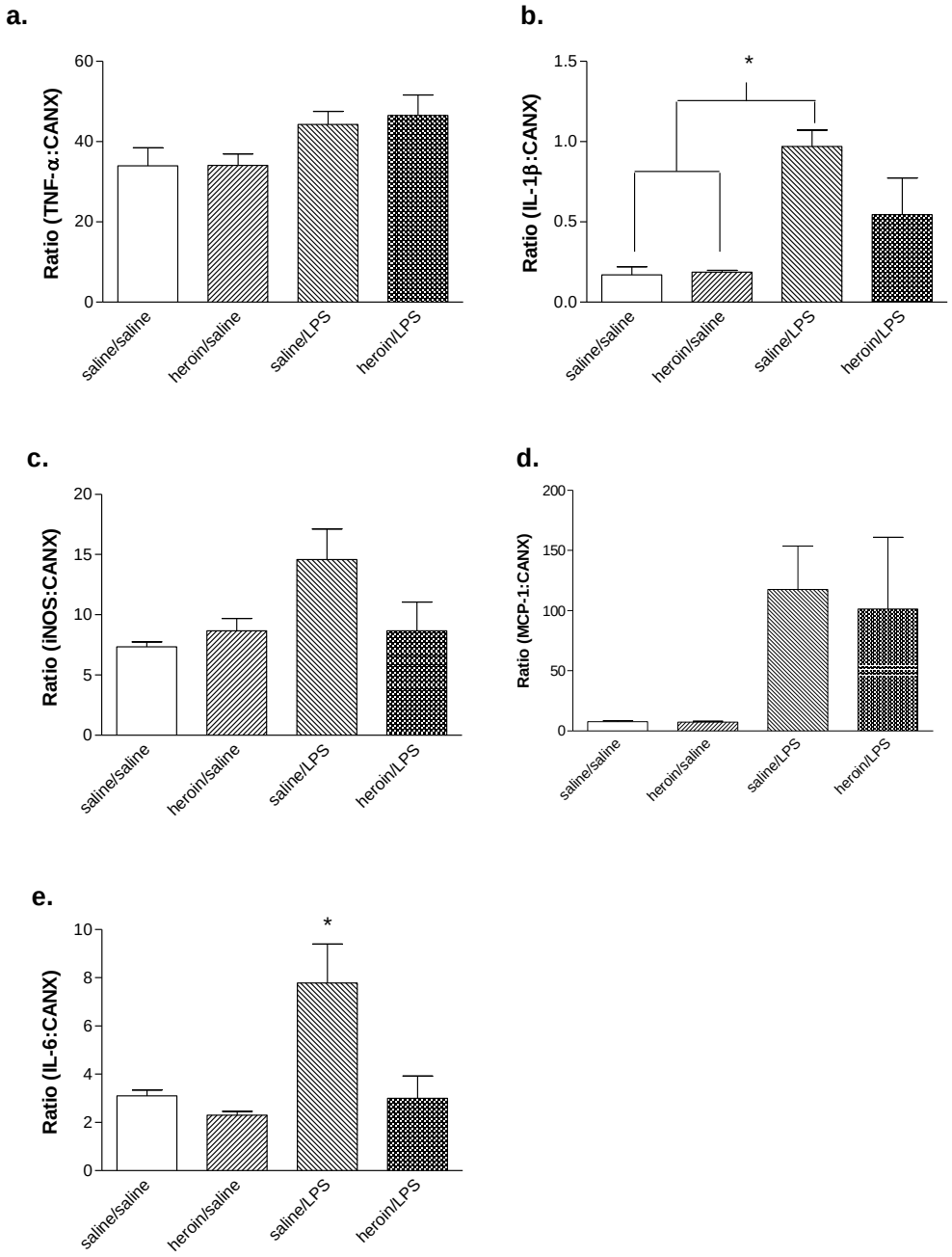


Figure 2.3 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 90 minutes after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graph represents the mRNA levels of **(a)** IκBα and **(b)** COX-2 measured in the hypothalamus and **(c)** IκBα and **(d)** COX-2 in the hippocampus.

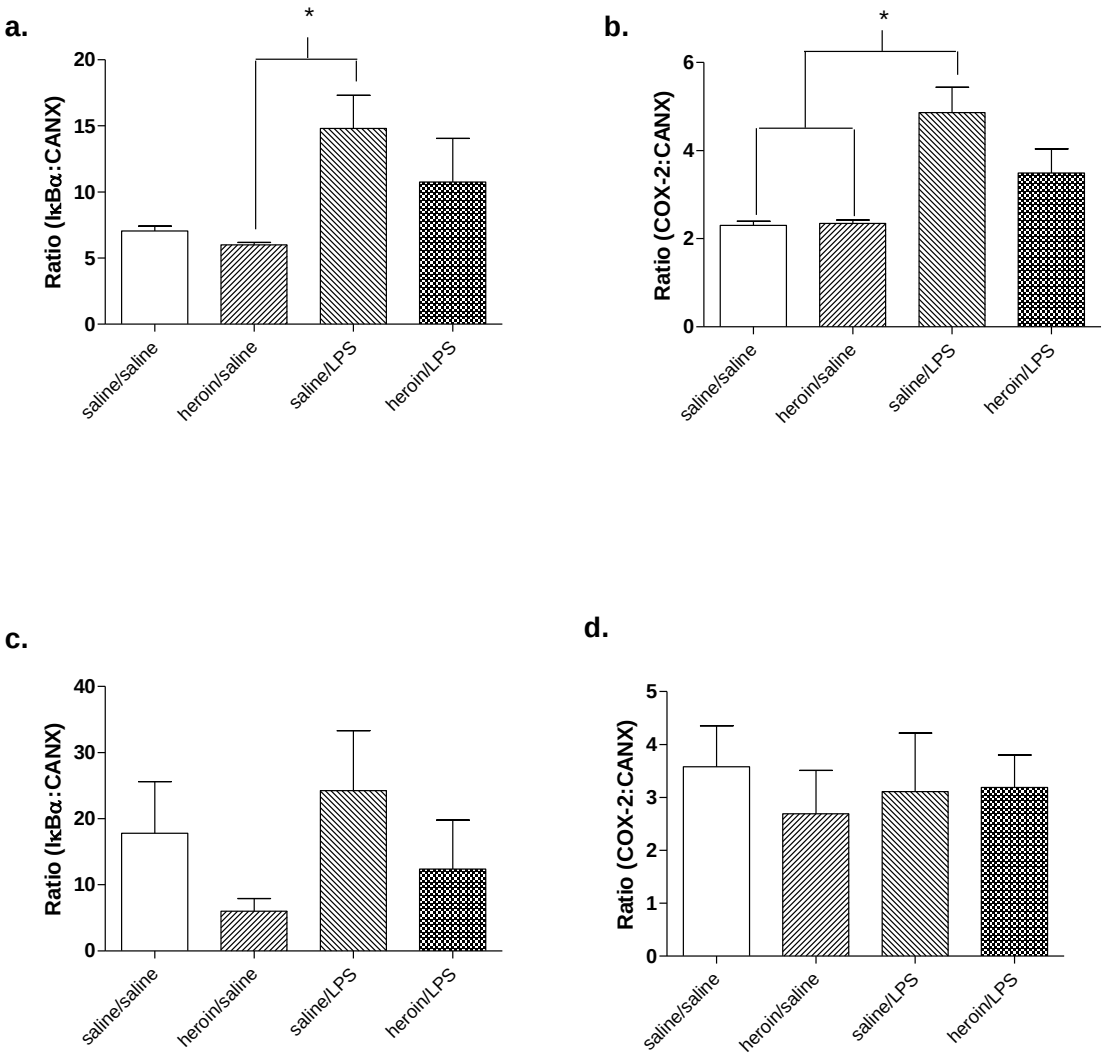


Figure 2.4 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 90 minutes after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels measured in the cortex. **(a)** TNF-α **(b)** IL-1β **(c)** iNOS **(d)** IL-6 **(e)** MCP-1

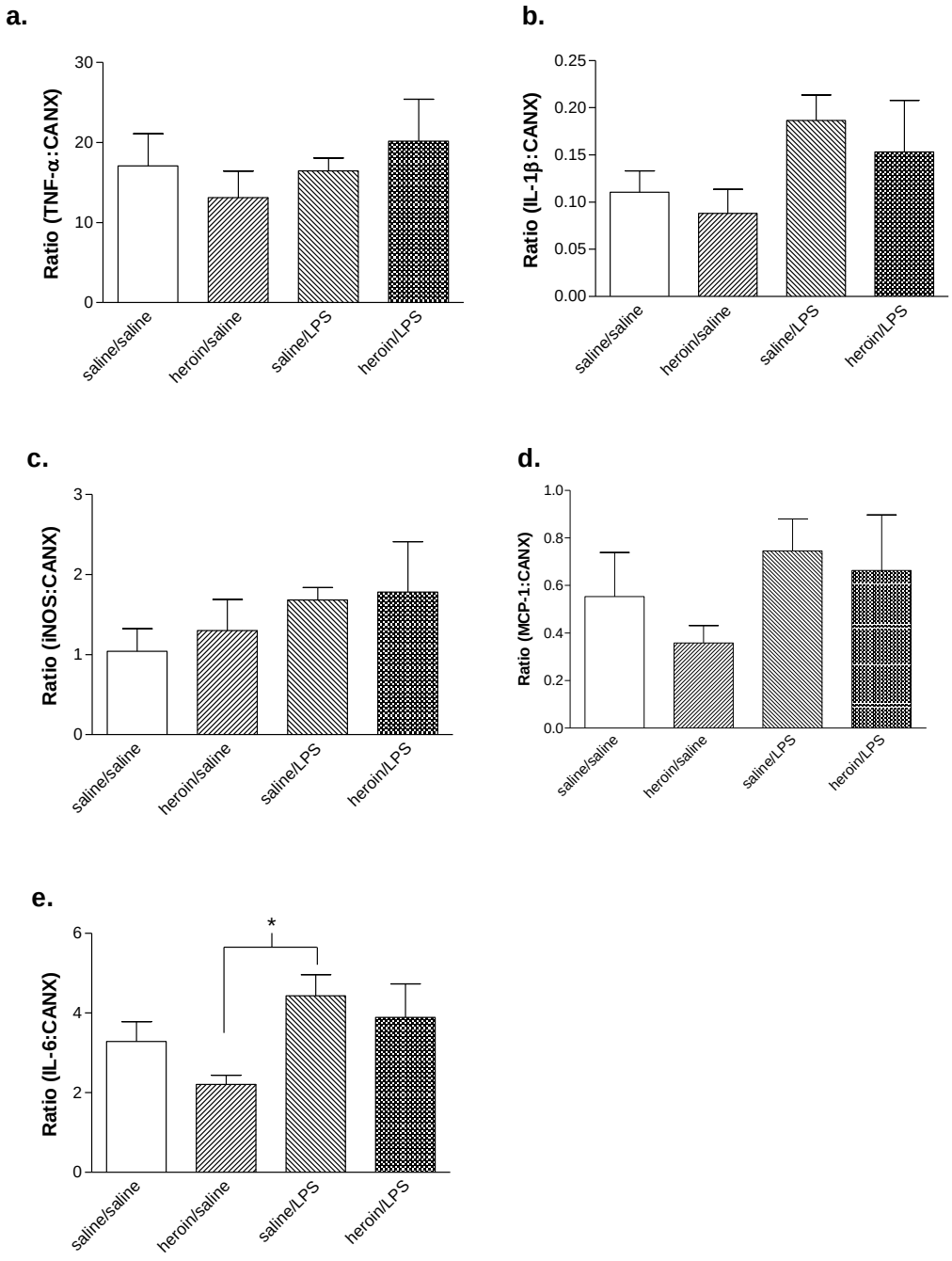
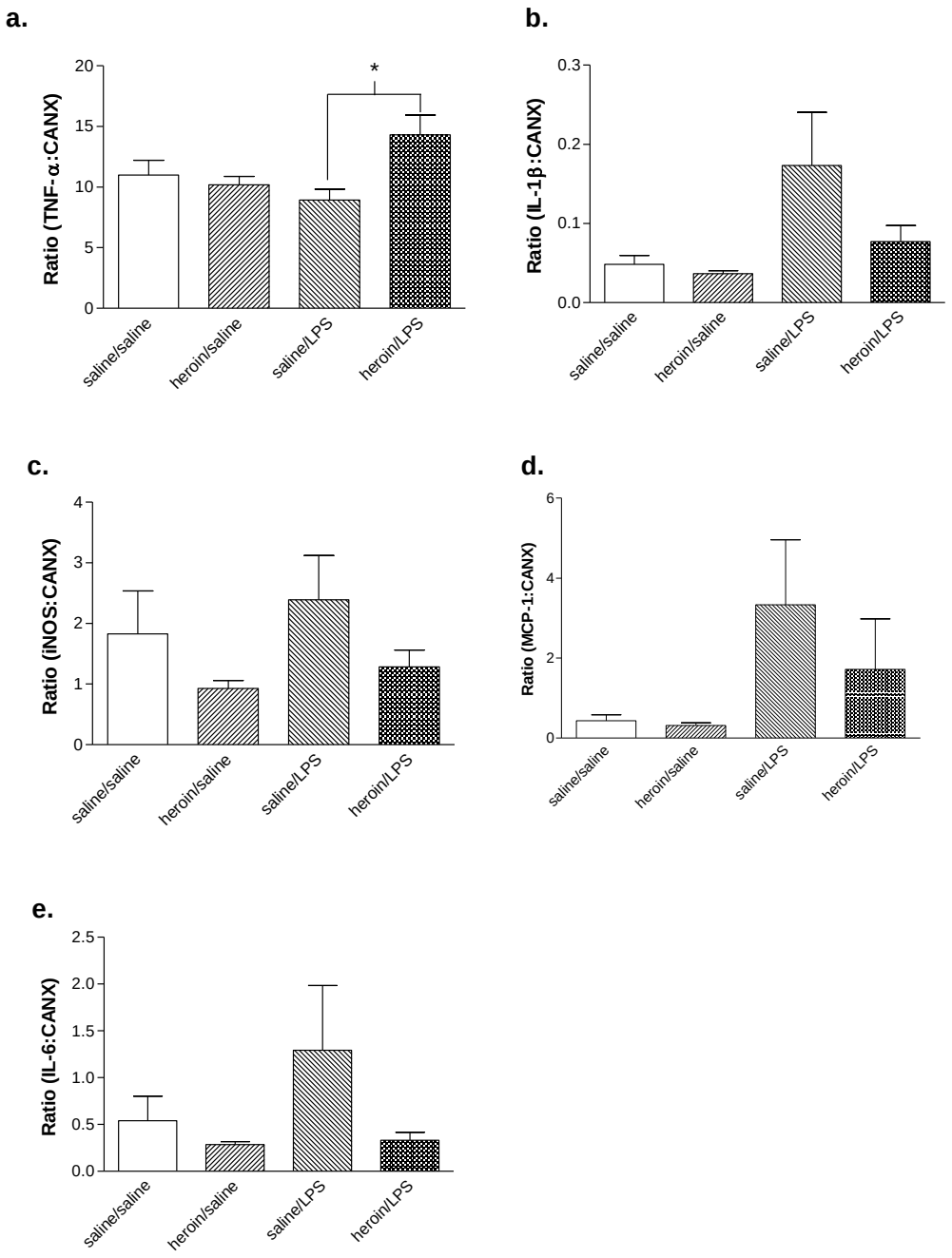


Figure 2.5 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 90 minutes after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels measured in the striatum. **(a)** TNF-α **(b)** IL-1β **(c)** iNOS **(d)** IL-6 **(e)** MCP-1



Chapter 3

Heroin produces suppressive effects on proinflammatory mediators in the hypothalamus and subsequent febrile response.

Introduction

In addition to the body temperature effects of opiates alone, it has also been reported that opiates such as morphine can inhibit fever production to LPS. Fever production is regulated by the hypothalamus and has been studied extensively. Although there are alternate pathways proposed that likely exist, a traditional or classical fever pathway has been well-defined for many years. Fever (also referred to as the febrile response) is discrete from general hyperthermia. The fever response follows a distinct pathway initiated by a complex series of events beginning with a perceived immune challenge that typically requires several hours to initiate due to the necessary biological mechanisms that must be performed. Hyperthermia, on the other hand, can be caused by any number of mechanisms, and is considered to be a more general term- fever is a type of hyperthermia, but not all hyperthermic responses are fevers. The previous studies from Chapter 2 clearly indicate that heroin induces one of these such non-fever hyperthermias.

As mentioned before, fever is a metabolically costly response that clearly must serve some evolutionarily adaptive function. Indeed, a fever response to infection is conserved throughout the evolutionary tree of most animals, including mammals, reptiles, birds, and even mollusks (Kluger, 1978). While the usefulness of fever can be dependent upon the type and duration of infection, one mechanism through which fever is beneficial is that increased body temperature serves to aid leukocytes in killing bacteria (Sebag et al., 1977). Blockade of fever has unfavorable effects on clearance of bacteria and detrimentally affects mortality rates (Bernheim & Kluger, 1976; Kluger, Ringler, & Anver, 1975).

Proinflammatory cytokines are key initiators of the fever response. IL-1β, TNF-α, and IL-6 are the most studied of the proinflammatory cytokines in relation to fever (Kluger et al., 1995; Jansky et al., 1995). Administration of these cytokines into the hypothalamus, specifically the preoptic anterior region (POA), causes fever, and this can be reversed with specific antibodies or receptor antagonists for the cytokines (Conti et al., 2004; Fernandez-Alonso, Benamar, Sancibrian, Lopez-Valpuesta, & Minano, 1996; Myers et al., 1994; Cartmell, Luheshi, & Rothwell, 1999). Although many studies have examined the effect of proinflammatory cytokines inducing fever, few have measured endogenous proinflammatory mediator production in the hypothalamus during fever production (Jansky et al., 1995).

IL-1β, TNF-α, and IL-6 are well established as inducers of fever, as is PGE_2. PGE_2 is typically viewed as the obligatory molecule in the traditional fever pathway. This is well supported throughout the literature- in mice unable to produce PGE_2, IL-

1β, IL-6 and TNF-α are all produced in the brain in response to LPS, fulfilling the initial stage of the fever pathway. However, fever is never initiated in these mice, indicating that proinflammatory cytokine response is not dependent upon PGE_2, but fever production is (Nilsberth et al., 2009b). Inhibition of COX-2, the inducible enzyme responsible for synthesizing PGE_2, via pharmacological means or genetic manipulation abolishes the fever response to LPS or IL-1β (Scammell, Griffin, Elmquist, & Saper, 1998; Li, Goorha, Ballou, & Blatteis, 2003; Li et al., 1999; Steiner et al., 2005). The dependence of PGE_2 action on COX-2 indicates that PGE_2 must be induced and is not constitutively present. Thus, the traditional fever pathway consists of the following events: proinflammatory cytokines (IL-1β, TNF-α, and IL-6, usually) present in the hypothalamus, leading to the synthesis of PGE_2, which then alters thermosensitive neurons in the POA, leading to behaviors that cause fever (shivering, warmth-seeking, blood vessel alterations).

The μ receptor and fever

The μ receptor seems to play a variety of roles in the production of fever as well as the effects seen in Chapter 2. In addition to the effects demonstrated by Mayfield et al of morphine impairing fever, antagonists to the μ opioid receptor injected peripherally and directly into the POAH prevent fever induced by IL-6, TNF-α, and LPS (Benamar, Geller, & Adler, 2002; Benamar, Xin, Geller, & Adler, 2000; Blatteis, Xin, & Quan, 1991). Mice that are deficient in the μ opioid receptor do not exhibit fever in response to LPS (Benamar et al., 2005; Benamar, Yondorf, Barreto, Geller, & Adler, 2007). These studies indicate a potential role for the μ receptor in

fever production. Numerous studies have indicated that β-endorphin can increase body temperature, and blockade of the μ receptors in studies such as those above would also block this effect (Rezvani, Gordon, & Heath, 1982; Tsai, Lin, Wang, & Huang, 2003; Tseng & Li, 1980; Vescovi et al., 1993; Martin & Bacino, 1979). Like morphine and heroin, β-endorphin also causes hyperthermia at low doses but causes hypothermia in high doses (Rezvani et al., 1982). In this case, activation of the μ receptors normally activated by β endorphin might explain the ability of heroin to induce hyperthermia. Injection of β-endorphin in the POA of the hypothalamus increased body temperature in two peaks, but only the first rise was blocked by naloxone, while the other rise in body temperature was blocked by indomethacin (Martin et al., 1979). It is possible that heroin is acting on the same receptors as β-endorphin – μ opioid receptors – to produce a hyperthermic effect that is similar to the first rise seen with β-endorphin injection, while the second rise seen with β-endorphin is connected to its potential role in fever production. Drugs of abuse are well-known for hijacking pathways intended for adaptive processes such as motivation, so it is no surprise that heroin would also act in a similar manner as β endorphin, although it is not clear why heroin does not produce the second peak in body temperature like β-endorphin. The role of β-endorphin in the production of fever is not yet well-defined, although as mentioned above, antagonizing the μ receptor does impair fever production (Martin et al., 1979; Benamar et al., 2007; Benamar et al., 2000). The normal β-endorphin response has been shown to also be altered with heroin use, which may also affect the production of fever to LPS

(Vescovi, Coiro, Volpi, Giannini, & Passeri, 1992; Vescovi et al., 1989; Jezova, Vigas, Tatar, Jurcovicova, & Palat, 1985).

In the following experiments, we evaluate the effects of heroin on fever production in response to LPS. Half of the rats were examined for mRNA analysis of proinflammatory mediators (TNF-α, IL-1β, iNOS, IL-6, MCP-1, IκBα, and COX-2) in the hypothalamus, while the other half of the rats were used to analyze protein levels of β-endorphin and the precursor molecule POMC. The disruption of either of these arms would be may provide a mechanism for alterations of alter fever production by heroin.

Materials and Methods

Animals

Animals were housed under the same conditions and approvals as listed in Chapter 2. There were a total of 48 rats used for these experiments.

Surgical procedures

Surgical procedures were the same as listed above for implantations of biotelemetry devices. As before, the rats were allowed at least one week to recover before further experimental procedures were conducted. Home cages were placed and kept on top of the biotelemetry receivers.

Drug administration

All injections of heroin, LPS, or saline were given subcutaneously on the abdomen. LPS was E. coli derived and purchased from Sigma-Aldrich (St. Louis, MO).

Dissection procedures

Immediately following cervical dislocation at 8 hours post-treatment, brains were extracted rapidly dissected immediately. Target tissues were dissected using Paxinos and Watson as a guide (Paxinos et al., 2005). Half of the subjects were selected for mRNA analysis, while the other half were designated for protein analysis, spread evenly amongst all the groups. Tissues for mRNA analysis were stored in RNAlater (Ambion, Austin, TX) at -80°C until processed for RNA extraction. Tissues for protein analysis were stored in an anti-proteinase buffer at -80°C.

Real time reverse transcription polymerase chain reaction (RT-PCR)

The same procedures as reported previously in Chapter 2 were used to determine mRNA levels of proinflammatory mediators in the hypothalamus. Total RNA was extracted from each of these brain tissues using Tri-Reagent (Molecular

Research Center, Inc., Cincinnati, OH), following modified methods (Chomczynski et al., 1987). Quantification was performed as described before and cDNA was created for RT-PCR analysis. The cDNA was then measured in individual runs of RT-PCR as previously described. The target molecules for this analysis included the following: TNF-α, IL-1β, iNOS, IL-6, MCP-1, Ikβα, and COX-2.

Protein extraction and β-endorphin Western blot

For the β-endorphin protein determination, protein was extracted from whole hypothalamus tissue immediately following sacrifice via cervical dislocation. Protein was extracted using freeze/thaw lysis in sterile water containing antiproteinases. Total protein was determined spectrophotometrically (Model EL312 kinetic reader; Bio-Tek, Winooski, VT, USA) using Bio-Rad protein dye. A standard amount of protein (25µg) was loaded on a NuPage 3-8% Tris-acetate gel and electrophoretically separated and blotted to a presoaked polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) . The membrane was then blocked with 5% dry milk in a TBS-T buffer and reacted with a β-endorphin/Met-enkephalin/β-LSH polyclonal goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. The membrane was then washed thoroughly and incubated with cow anti-goat secondary antibody in 5% dry milk TBS-T. Membranes were then washed and exposed to chromagen for 2-60 minutes and scanned for quantitative analysis. Analysis was performed using Image Pro® Plus program (Media Cybernetics, Silver Spring, MD), which measures density of the dyed protein.

Data and Statistical Analysis.

Body temperature data analysis. Body temperature data was analyzed based upon samples made at every hour point that was averaged between five data points (e.g., for measurement of hour after treatment, data from the 58, 59, 60, 61, 62 minutes post-treatment were analyzed and represented the 1 hour data point for that particular rat). Body temperatures were followed until sacrifice of the rat via cervical dislocation at 8 hours after heroin treatment.

β-endorphin protein analysis. Bands density was quantified and reported as means, which was the result of total band density divided by band area.

Proinflammatory mediator mRNA data analysis. To ensure comparable quality of RNA among samples, the housekeeping gene calnexin (CANX) was assessed and target molecule data were expressed as a ratio to amount of CANX measured.

Statistics. All statistics were performed using SPSS v17.0. For body temperature data, repeated measures ANOVA was used to determine if there was a time * heroin interaction. If interactions were found, Tukey posthoc tests were performed. For mRNA and protein data, ANOVAs were performed examining the main effects and interactions for LPS and heroin for all proinflammatory mediators, and Tukey posthoc tests were also performed when significant effects were found. Significance was set at $p \leq 0.05$.

Results

Body temperature

Body temperature data was analyzed using a mixed-design repeated measures ANOVA with LPS/saline and heroin/saline treatments as independent variables and time (using hourly timepoints) as a repeated measure. Using this information, the ANOVA revealed a time * LPS * heroin interaction, [$F(8,344)=5.765$, $p \leq 0.001$]. Additionally, there was a between-subjects main effect of LPS [$F(1,43)=11.671$, $p \leq 0.001$]. Posthoc tests revealed a number of differences. For Hour 1, the heroin/saline treated group was significantly elevated compared to the saline/saline treated group ($p \leq 0.05$), indicating that the heroin-induced hyperthermia occurred, which is consistent with the previous findings in Chapter 2. At Hour 2, the saline/LPS treated group had a significantly lowered body temperature compared to the saline/saline ($p \leq 0.001$), heroin/saline ($p \leq 0.001$), and heroin/LPS ($p \leq 0.001$) treated groups, which is a normal part of the initial effects of LPS. There were no significant differences in any groups for Hour 3. Hour 4 posthoc tests revealed only one significant difference, between the heroin/saline treated group and the heroin/LPS treated group ($p \leq 0.01$). By Hour 5, fever production had begun and the saline/LPS treated group was significantly elevated compared to the saline/saline ($p \leq 0.01$) and heroin/saline ($p \leq 0.001$) treated groups, while the heroin/LPS treated group only differed from the heroin/saline treated group ($p \leq 0.001$). At Hour 6, the saline/LPS treated group continued to be significantly elevated compared to saline/saline ($p \leq 0.001$) and heroin/saline ($p \leq 0.001$) treated groups, as does the

heroin/LPS treated group ($p \leq 0.001$ for both). Using posthoc tests, the saline/LPS treated group did not reach significance compared to the heroin/LPS treated group, but an *a priori* t-test revealed a significant difference between the two [$t(22)=2.334$, $p \leq 0.05$]. By Hour 7, the saline/LPS treated group was significantly different than all of the other groups: saline/saline ($p \leq 0.001$), heroin/saline ($p \leq 0.001$), and heroin/LPS ($p \leq 0.05$). The heroin/LPS treated group also differed significantly from the heroin/saline treated group ($p \leq 0.01$), and although nonsignificant results are generally not reported throughout this document, it is notable to point out that the heroin/LPS group, at Hour 7, does not differ significantly from the saline/saline treated group ($p=0.637$). By Hour 8, the saline/LPS treated group no longer differs significantly from the heroin/LPS treated group, although it does still retain elevations compared to the saline/saline ($p \leq 0.01$) and heroin/saline ($p \leq 0.01$) treated groups. **Figure 3.1**

β-endorphin protein analysis

Pilot studies (not shown) were performed to ensure validity of β-endorphin Western protocol. The pilot results indicated that β-endorphin could be measured and differences between samples with differing amounts of protein could be determined. For the present experiment, analysis was run on both β-endorphin and POMC, and means of density were examined. There were no significant main effects of LPS or heroin and no interactions of LPS or heroin. **Figure 3.2**

RT-PCR

In the hypothalamus, 2 (heroin tx) x 2 (LPS tx) ANOVA found a main effect of LPS on levels of TNF-α [$F(1,19)=17.64$, $p\leq0.01$], IL-1β [$F(1,19)=10.68$, $p\leq0.01$], IL-6 [$F(1,19)=24.64$, $p\leq0.001$], iNOS [$F(1,19)=13.66$, $p\leq0.01$], MCP-1 [$F(1,19)=7.94$, $p\leq0.05$], IκBα [$F(1,19)=20.26$, $p\leq0.01$], and COX-2 [$F(1,19)=24.01$, $p\leq0.01$]. A main effect of heroin was found on levels of TNF-α [$F(1,19)=7.01$, $p\leq0.05$], IL-1β [$F(1,19)=10.67$, $p\leq0.01$], IL-6 [$F(1,19)=20.17$, $p\leq0.001$], iNOS [$F(1,19)=6.98$, $p\leq0.05$], IκBα [$F(1,19)=16.16$, $p\leq0.01$] and COX-2 [$F(1,19)=4.914$, $p\leq0.05$]. An interaction between LPS and heroin was found on levels of IL-6 [$F(1,19)=23.01$, $p\leq0.001$], iNOS [$F(1,19)=4.85$, $p\leq0.05$] and IκBα [$F(1,19)=6.86$, $p\leq0.05$]. Posthoc tests revealed that the LPS/saline treated group was significantly elevated compared to all other groups in mRNA levels of TNF-α, IL-1β, IL-6, iNOS, IκBα, and COX-2, and the LPS/saline treated group differed significantly from the saline/saline and saline/heroin groups in levels of MCP-1.

These results indicate that LPS induces a clear proinflammatory response in the hypothalamus which included all molecules measured, and with the exception of MCP-1, heroin suppresses each of these proinflammatory mediators to such an extent that they are no longer different than the saline/saline or heroin/saline treated groups. **Figure 3.3 and 3.4**

Discussion

Major findings

The results of these experiments clearly demonstrate that LPS induces proinflammatory mediator mRNA in the hypothalamus. Heroin, in contrast, has a suppressive effect on production of proinflammatory mediators and COX-2, but not β-endorphin, in the hypothalamus after stimulation with LPS, and this is correlated with an attenuation of fever response.

LPS induces proinflammatory mediator mRNA in the hypothalamus

In the present results, LPS significantly elevated all proinflammatory mediators in the hypothalamus. This is consistent with the vast literature implicating proinflammatory mediators in fever production. In most published studies examining proinflammatory mediators, protein is the measure used. The results of the experiments in this chapter are complementary to these studies, as now it can be established that these proinflammatory are not only *present* in the hypothalamus, but also being *actively produced* in the hypothalamus. Few studies thus far have used examination of mRNA to study these processes related to fever, and none have looked at multiple proinflammatory mediators. This may seem a minute point, but there has been a great deal of discussion regarding the source of proinflammatory cytokines- whether they are trafficked in from the periphery or made within the hypothalamus (Maier et al., 1998b). By now, most scientists favor the hypothesis that the cytokines are made within the hypothalamus due to the inability of cytokines to cross the blood brain barrier easily; however, there is little conclusive proof for this. It is likely that there are a number of ways that the brain is signaled to begin

producing proinflammatory mediators, the most likely being that the vagal nerve transmits information from immune organs to stimulate proinflammatory cytokines in the brain (Blatteis, Sehic, & Li, 2000; Blatteis et al., 2005; Maier, Goehler, Fleshner, & Watkins, 1998a). While the experiments presented here are not designed to rule out other mechanisms for cytokines to be present in the hypothalamus, the results do indicate that regardless of signal origin, cells within the hypothalamus are producing proinflammatory cytokines (TNF-α, IL-1β, IL-6, MCP-1) and other mediators (iNOS, Iκβα, COX-2), and these cytokines are correlated with the production of fever. Further discussion into the potential pathways of signaling is found in Chapter 5.

Heroin suppresses proinflammatory mediator mRNA in the hypothalamus

The results of this experiment show that heroin suppresses the mRNA of proinflammatory mediators in the hypothalamus. Although Mayfield et al (1998) demonstrated that morphine suppresses plasma IL-1β and TNF-α, no studies to date have examined the effects of opiates on proinflammatory mediators within the hypothalamus. Together with the data from Chapter 2 and 4, this is the first study to evaluate the effects of opiates on in-brain cytokines. The suppressive effects of heroin are very clear in all measures of the proinflammatory mediator mRNA data presented here. While it has been established that opiates suppress peripheral immune responses and that suppression is at least partially mediated through neural

communications, this is the first to examine how heroin might alter immune responses within the brain and effects of these alterations.

Lack of involvement of β-endorphin in alterations produced by heroin

The lack of changes in β-endorphin in the hypothalamus was an unexpected effect, given that β-endorphin is likely to play a role in the production of fever and heroin users have altered β-endorphin responses in addition to altered infection rates. However, upon closer examination, there may be reasonable explanations for this occurrence. The first and most logical point to make is that this experiment only examined one time point, 8 hours post-treatment. It is entirely possible that β-endorphin increases are transient and occur prior to the timepoint examined. Another important point is that while the experiment was intended to study acute effects of heroin on fever production and related molecular mediators, the studies indicating that heroin users had an altered β-endorphin response were conducted on users that regularly abused heroin (Vescovi et al., 1992; Vescovi et al., 1989). The chronic effects versus acute effects of heroin are quite different, and perhaps β-endorphin responses are altered after chronic use of heroin as opposed to a more acute effect (Wang et al., 2008; Mocchetti et al., 1989). That said, it is also possible that neither heroin nor LPS actually affect β-endorphin under any conditions, although that would argue against the number of studies implicating the μ opioid receptor in fever production as a response to LPS. In fact, one study found that intracerebroventricular injection of LPS, IL-1β, or PGE_2 increased staining for β-

endorphin; it is not clear if the difference between systemic (as in this study) and intracerebral administration altered the outcome of β-endorphin (Tsai et al., 2003). Regardless, β-endorphin is not part of the *traditional* fever pathway, and it is unlikely that a lack of effect on β-endorphin would undermine any other effects seen in the molecules implicated in the traditional fever pathway such as cytokines and PGE_2.

Suppression of fever response by heroin

The ability of heroin to suppress the proinflammatory mediators in the hypothalamus is reflected in the concurrent attenuation of fever response. We have established that the hyperthermia induced by heroin is not related to production of proinflammatory mediators in the hypothalamus, and that the administration of heroin attenuates the production of fever in response to LPS. It can be concluded, then, that the attenuation of fever is not directly related to the earlier hyperthermia; that is, the brief hyperthermia induced by heroin does not exhaust the potential to produce proinflammatory cytokines and prevent fever in this manner. However, the suppression of proinflammatory cytokines by heroin during fever is quite apparent in both the cytokine (TNF-α, IL-1β, IL-6, MCP-1) levels seen as well as the potential for PGE_2 induction (COX-2). The disruption of this pathway predictably results in an inhibition of fever response. The lack of fever response in rats treated with heroin may be one mechanism through which heroin users are less able to fight off infection. It also represents the importance of proinflammatory responses in the brain, and highlights the consequences of suppression of these responses.

Conclusion

The results presented here represent the first efforts to determine the consequences of opiates on the immune response within the brain. These results show that heroin suppresses brain proinflammatory mediator mRNA in the hypothalamus, and a concurrent suppression of fever is also seen. Future studies to elaborate on these results and address the potential mechanisms through which opiates might exert these effects are discussed further in Chapter 5.

Table 2. Significance values by group for the duration of the 8-hour experiment measuring core body temperature after 1mg/kg heroin or saline and 1mg/kg LPS or saline treatment. ** t test comparing saline/LPS and heroin/LPS treated groups revealed a significant difference (p=0.029).

		Baseline	Hour 0	Hour 1	Hour 2	Hour 3
saline/saline						
	heroin/saline	0.902	0.541	0.037	0.167	0.858
	saline/LPS	0.655	0.523	0.535	0.001	0.527
	heroin/LPS	0.949	0.934	0.102	0.685	0.928
heroin/saline						
	saline/saline	0.902	0.541	0.037	0.167	0.858
	saline/LPS	0.962	0.039	0.463	0.001	0.935
	heroin/LPS	0.999	0.212	0.969	0.739	0.484
saline/LPS						
	saline/saline	0.655	0.523	0.535	0.001	0.527
	heroin/saline	0.964	0.039	0.463	0.001	0.935
	heroin/LPS	0.920	0.855	0.737	0.001	0.196
heroin/LPS						
	saline/saline	0.949	0.934	0.102	0.685	0.928
	heroin/saline	0.999	0.212	0.969	0.739	0.484
	saline/LPS	0.920	0.855	0.737	0.001	0.196
		Hour 4	**Hour 5**	**Hour 6**	**Hour 7**	**Hour 8**
saline/saline						
	heroin/saline	0.153	0.332	0.922	0.132	0.849
	saline/LPS	0.981	0.004	0.001	0.001	0.030
	heroin/LPS	0.440	0.082	0.001	0.637	0.581
heroin/saline						
	saline/saline	0.153	0.332	0.922	0.132	0.849
	saline/LPS	0.280	0.001	0.001	0.001	0.003
	heroin/LPS	0.003	0.001	0.001	0.006	0.157
saline/LPS						
	saline/saline	0.981	0.004	0.001	0.001	0.030
	heroin/saline	0.280	0.001	0.001	0.001	0.003
	heroin/LPS	0.231	0.605	0.073**	0.033	0.371
heroin/LPS						
	saline/saline	0.440	0.082	0.001	0.637	0.581
	heroin/saline	0.003	0.001	0.001	0.006	0.157
	saline/LPS	0.231	0.605	0.073	0.033	0.371

Figure 3.1 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means by treatment group with standard error of means (SEM). Body temperature was measured via 1 minute samples taken over 5 minutes and averaged per rat at every hour interval. * denotes significance of at least p≤0.05.

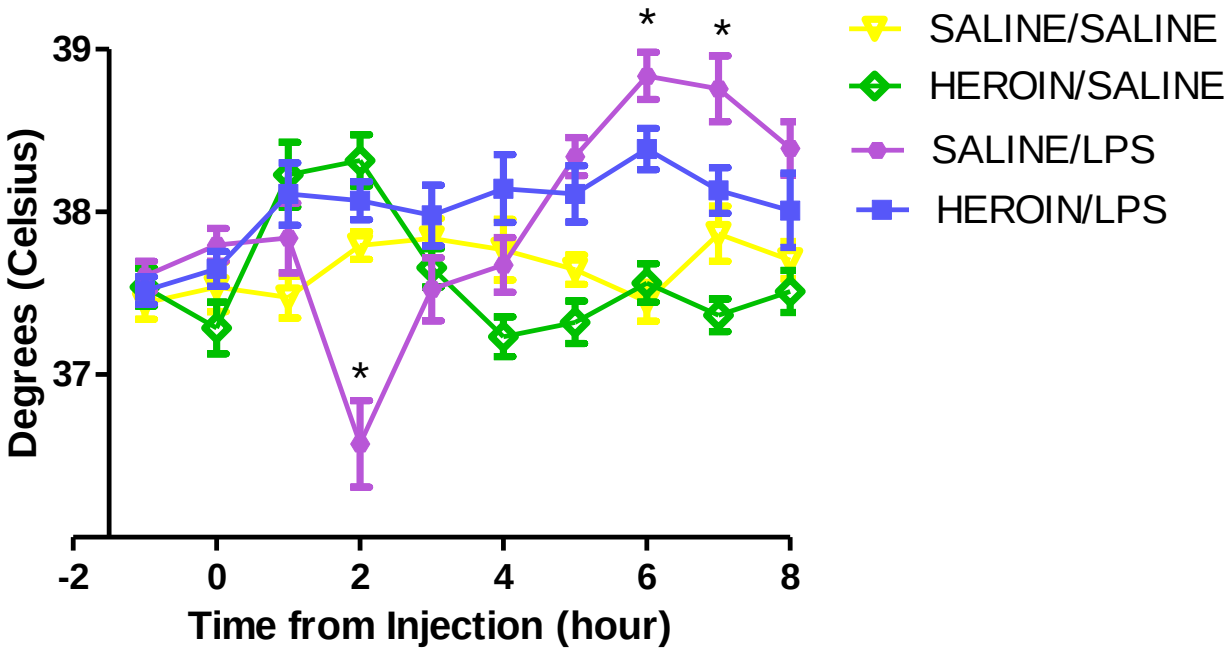
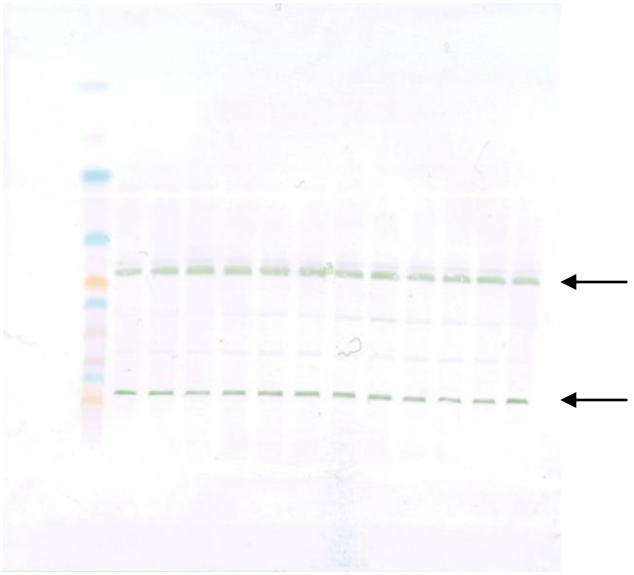
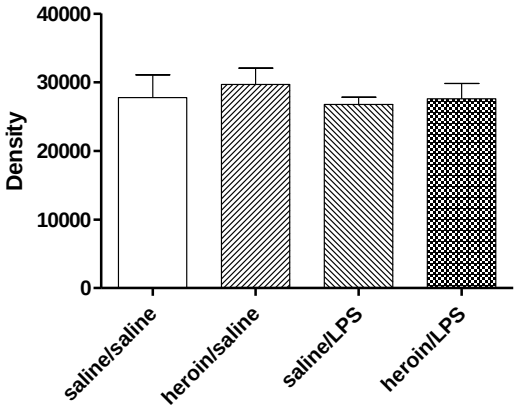


Figure 3.2 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means by treatment group with standard error of means (SEM). β endorphin and POMC were measured from protein derived from dissected hypothalamus via Western blot. **(a)** Picture of actual β-endorphin Western blot. The top row represents POMC while the lower row indicates β-endorphin. Density was quantitated by computer and is expressed by a ratio of total density by total area for POMC **(b)** and β-endorphin **(c)**. Data is represented as means by treatment group with standard error of means (SEM).

a.



b.



c.

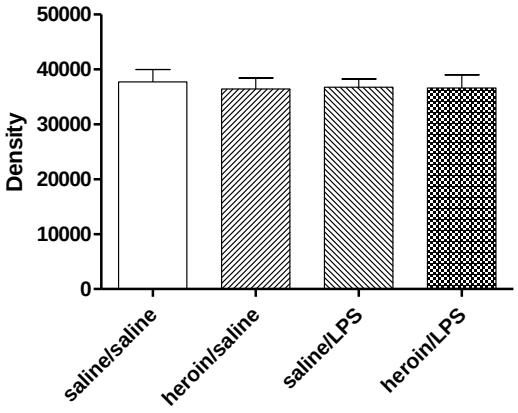


Figure 3.3 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels measured in the hypothalamus. **(a)** TNF-α **(b)** IL-1β **(c)** iNOS **(d)** IL-6 **(e)** MCP-1

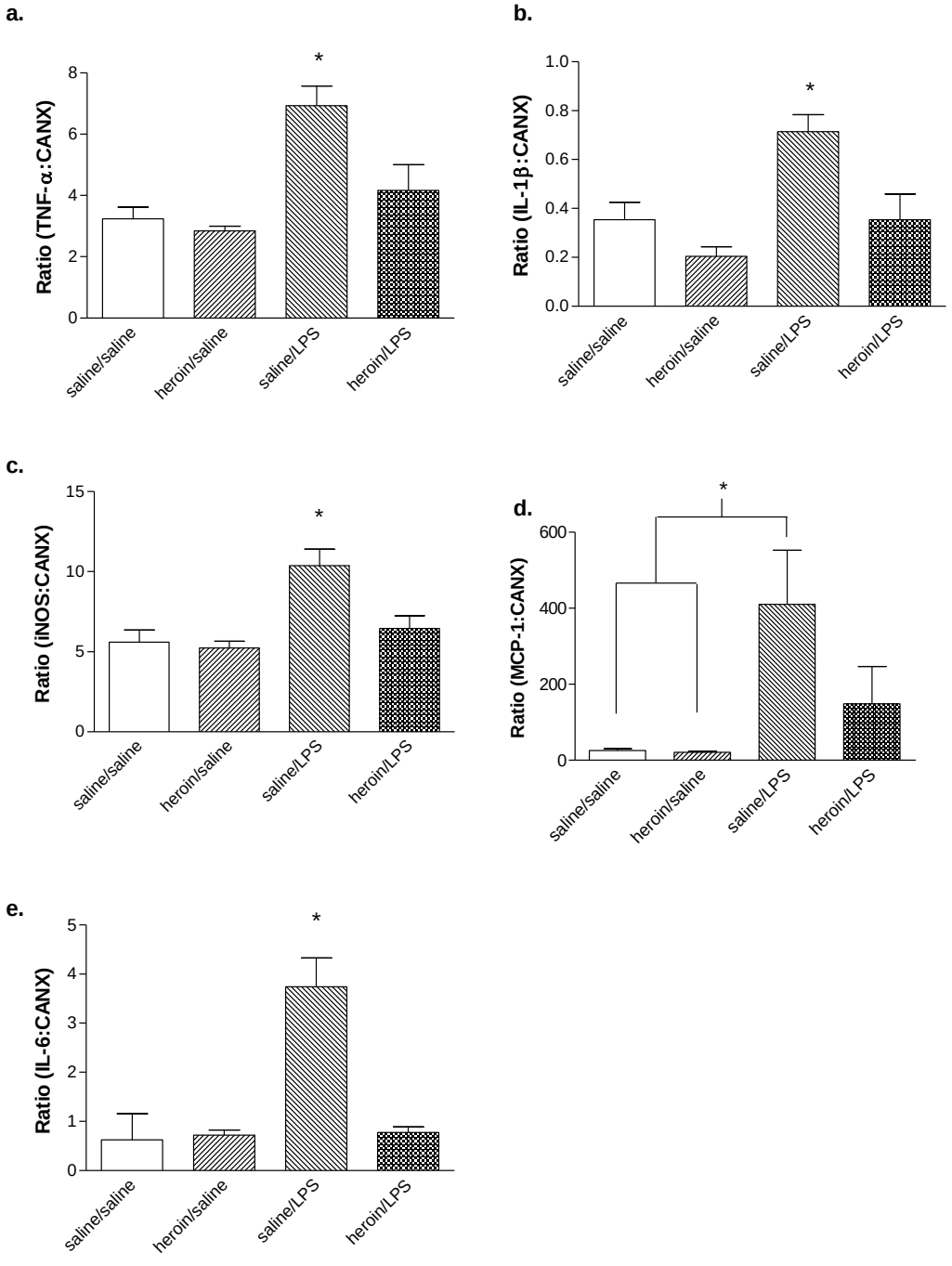
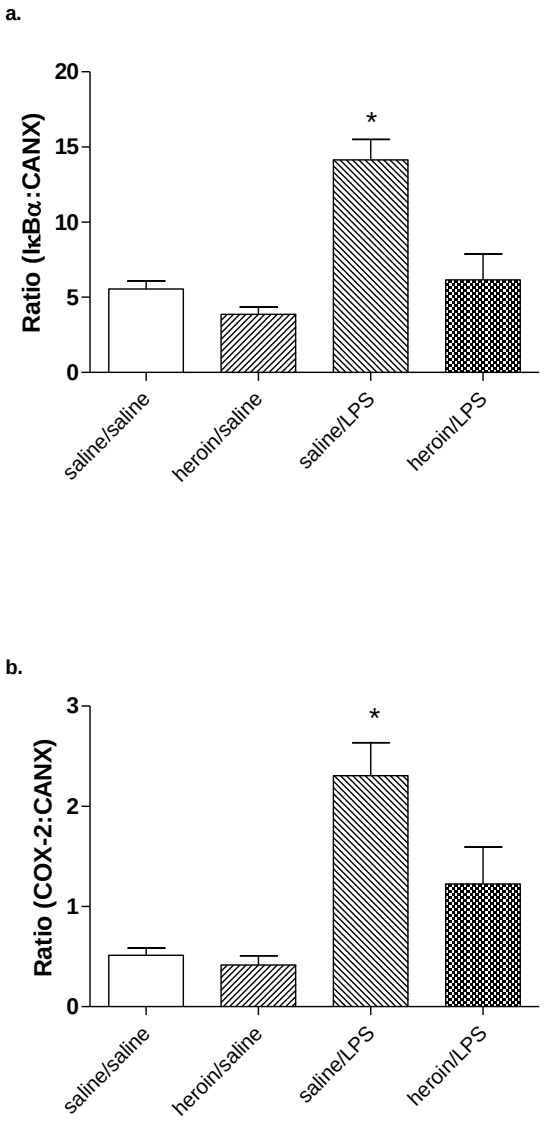


Figure 3.4 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least $p \leq 0.05$. The following graphs represent the mRNA levels of **(a)** IκBα and **(b)** COX-2 measured in the hypothalamus.



Chapter 4

Heroin suppresses LPS-induced proinflammatory mediators in the hippocampus and subsequent behavioral depression.

Introduction

Proinflammatory mediators such as TNF-α, IL-1β, iNOS, IL-6, and MCP-1 are commonly found preferentially in the hippocampus after various types of immune stimulation (Semmler, Okulla, Sastre, Dumitrescu-Ozimek, & Heneka, 2005; Wolff et al., 2009). This is likely to be a result of high numbers of microglia in the hippocampus (Lawson, Perry, Dri, & Gordon, 1990). Immune activation induces a change in motivational state that results in a decrease in activity requiring increased proinflammatory mediators as a vital part of this process. In the field of neurobiology, the hippocampus is a brain region typically associated with learning and memory. The presence and synthesis of these proinflammatory mediators in the hippocampus results in learning and memory functions normally regulated by this region being largely disrupted. There is a great deal of overlap between the literature implicating proinflammatory mediators in the hippocampus with hippocampally-dependent learning impairments and a reduction in activity as a sickness behavior.

Learning and memory deficits during immune activation are well documented. The specificity of the hippocampal inflammation and its detrimental effects on learning and memory are seen in a set of experiments in which rats administered LPS during the consolidation period after conditioning displayed an impairment in learning for hippocampally dependent context conditioning but not for hippocampus-independent auditory fear conditioning (Pugh et al., 1998; Rachal, Fleshner, Watkins, Maier, & Rudy, 2001). In these experiments, administration of IL-1 receptor antagonist reversed the impairments produced by LPS on context conditioning, indicating a causal role for IL-1β. However, IL-1β is not the only proinflammatory mediator linked to increases during immune activation in the hippocampus and subsequent learning and memory deficits. In addition to proinflammatory cytokines, iNOS is preferentially increased in the hippocampus compared to a number of other brain regions (Semmler et al., 2005). IL-6 also plays an important role that is specific to brain inflammatory responses. IL-6 knockout mice display a normal TNF-α and IL-1β response in the peripheral system after LPS stimulation; however, these mice have an attenuated IL-1β and TNF-α response in hippocampal tissue, indicating the necessity of IL-6 in order for this pathway to come to completion (Sparkman et al., 2006). In addition to the molecular alterations seen in these mice, the IL-6 knockout mice did not demonstrate deficits in working memory after LPS treatment, contrary to their IL-6 (+/+) counterparts. Additionally, interleukin-10 (IL-10), a typically anti-inflammatory cytokine, deficit mice had a prolonged inflammatory response in the hippocampus and a more severe deficit in ability to integrate new information compared to mice with normal IL-10 responses

(Richwine et al., 2009). IL-1β and TNF-α also decrease glutamatergic uptake in the hippocampus as well as cause an impairment of long-term potentiation (LTP), which suggests a potential mechanism for these learning impairments (Tanaka et al., 2006; Hauss-Wegrzyniak, Lynch, Vraniak, & Wenk, 2002; Cunningham, Murray, O'Neill, Lynch, & O'Connor, 1996).

Aging causes increased proinflammatory mediators in the hippocampus as an independent effect, and these effects are potentiated by LPS injection, causing increased IL-1β up to 24 hours later and behavioral depression; these effects can be ameliorated by antagonizing IL-1β (Abraham & Johnson, 2009). A prolonged inflammatory response to immune challenge has been seen in aging rodents in a number of experiments, and appears to be specific to the hippocampus and hippocampally-dependent tasks such as context and spatial learning (Barrientos, Watkins, Rudy, & Maier, 2009; Barrientos et al., 2009). Increased IL-1β in the hippocampus is also seen after LPS administration that disrupted working memory in aged rats (Chen et al., 2008).

The learning and memory impairments produced by an inflammatory response in the hippocampus have clearly been demonstrated. However, simply impairing learning abilities is probably not in itself an evolutionarily adaptive behavior to exhibit during illness and is more likely to be a side effect of initiation of a more adaptive behavior. Behavioral depression, demonstrated by an avoidance of social contact and general activity, is an adaptive behavior to exhibit during illness. The lack of activity allows more energy reserves to be directed towards activating the

immune system to effectively clear the invading infection from the body, as well as to increase body temperature.

The cytokines and other proinflammatory mediators shown to produce learning and memory deficits are the same as those associated with behavioral depression during sickness. Given that, there is predictably a significant overlap between learning deficits and production of sickness behaviors. The production IL-1β, in particular, has been linked to sickness behavior (Campisi et al., 2003). IL-6 and TNF-α are also believed to play a role in sickness behavior ilicited from the hippocampus, although the roles of these cytokines are not as well defined as IL-1β (Henry et al., 2008; Anisman et al., 2008; Pauli et al., 1998). Similar to learning and memory deficits, aged mice have a more robust behavioral sickness response to LPS which can be reversed with central administration of IL-1 receptor antagonists (Abraham et al., 2009). Prolonged cytokine production in the hippocampus due to a deficiency of the anti-inflammatory cytokine IL-10 was also correlated with longer demonstration of sickness behavior (Richwine et al., 2009). LPS induces increased levels of IL-1β and IL-6 in the hippocampus and cortex that is correlated with behavioral depression; reversal of these cytokines and behavioral depression can be achieved with inhibition of microglial activation, indicating both of these effects are dependent upon activation of microglia induced by LPS (Henry et al., 2008). Although the connection is not as thoroughly established as fever is with the hypothalamus, proinflammatory cytokines are an imperative part of the pathway to induce behavioral depression, and the hippocampus has been implicated as a key

region for this phenomenon. The following experiments help to further establish the role of the hippocampus in behavioral depression as measured by activity.

Opiates such as morphine and heroin have been shown to suppress peripheral proinflammatory cytokines, an effect that may be related to the increased infection rates in opiate users. However, no studies have examined the effects of systemic opiates on brain proinflammatory cytokines (except those already presented in this dissertation), particularly in the context of the hippocampus and behavioral depression. The purpose of the following studies is to examine how heroin affects the cytokine portion of the sickness pathway in the hippocampus, and consequences on behavioral depression. The following experiment consisted of administration of concomitant LPS or saline and heroin or saline and examination of proinflammatory mRNA in the brain 8 hours post-treatment. This experiment demonstrated that heroin has an inhibitory effect on the induction of proinflammatory mediator mRNA by LPS in the hippocampus, as well as a suppression of behavioral depression. Since there is less research regarding the hippocampus and behavioral depression, cortex and striatum tissue was also examined for reference, although direct comparisons are not possible between the tissues due to limitations in PCR runs.

Materials and Methods

Animals

Animals were housed under the same conditions and approvals as listed in Chapter 2. There were a total of 48 rats used for these experiments.

Surgical procedures

Surgical procedures were the same as listed previously in Chapter 2 for implantations of biotelemetry devices. As before, the rats were allowed at least one week to recover before further experimental procedures were conducted. Home cages were placed and kept on top of the biotelemetry receivers.

Drug administration

All injections of heroin, LPS, or saline were given subcutaneously on the abdomen. LPS was E. coli derived and purchased from Sigma-Aldrich (St. Louis, MO).

Dissection procedures

Immediately following cervical dislocation, brains were extracted rapidly dissected immediately. Target tissues were dissected using Paxinos and Watson as a guide (Paxinos et al., 2005). Half of the subjects were selected for mRNA analysis, while the other half were designated for protein analysis, spread evenly amongst all the groups. Tissues for mRNA analysis were stored in RNAlater

(Ambion, Austin, TX) at -80°C until processed for RNA extraction. Tissues for protein analysis were stored in an anti-proteinase buffer at -80°C.

Real time reverse transcription polymerase chain reaction (RT-PCR)

The same procedures as reported previously were used to determine mRNA levels of proinflammatory mediators in the hypothalamus. Total RNA was extracted from each of these brain tissues using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH), following modified methods described by (Chomczynski et al., 1987). Quantification was performed as described before and cDNA was created for RT-PCR analysis. The cDNA was then measured in individual runs of RT-PCR as previously described. The target molecules for this analysis included the following: TNF-α, IL-1β, iNOS, IL-6, MCP-1, Ikβα, and COX-2.

Data and Statistical Analysis

Statistical analysis. All analyses were performed using SPSS v17.0. Significance was set at p=0.05. If significant results for Levene's test of equality for variances were noted, equal variances were not assumed for results. Tukey's posthoc tests were used when main effects or interactions were found to be significant.

RT-PCR. Data were converted to ratios based on the housekeeping gene CANX to ensure the most accurate comparisons. Housekeeping gene CANX was

assessed using the raw copy numbers to ensure that no significant differences were found between groups in this important baseline measure. A 2 (LPS treatment) x 2 (heroin treatment) ANOVA was run for each target molecule.

Activity. Activity data was transmitted and recorded by a nearby computer in one minute bins. Bins were summed over each hour for each rat. A time * LPS * heroin mixed design ANOVA was run with time as a repeating measure and LPS and heroin as independent variables. Another 2 (LPS treatment) x 2 (heroin treatment) ANOVA was also run to determine overall main effects and interactions of LPS and heroin independent of time. This analysis consists of binned data over the entire 8 hour time period that occurred between treatment and sacrifice.

Results

Activity

To determine if there was an interaction with time for the measure of activity, a repeated measures ANOVA was used with summed bins of activity per hour for each rat. LPS and heroin treatments were independent variables while time (with 9 time points) served as a repeated measure. For this analysis, the interaction between time * LPS * heroin was significant [$F(8,344)=2.720$, $p \leq 0.01$]. Additional analysis indicated a between subjects main effect of LPS [$F(1,20)=89.690$, $p \leq 0.001$] and a significant interaction of heroin and LPS [$F(1,20)=27.946$, $p \leq 0.001$]. For full posthoc test results, refer to Table 3 (page 71). To summarize the most striking findings, at Hour 1, the heroin/saline and heroin/LPS treated groups were

significantly decreased compared to the saline/saline ($p \leq 0.001$, $p \leq 0.001$) and saline/LPS ($p \leq 0.001$, $p \leq 0.001$) treated groups. By Hour 2, the saline/LPS treated group was significantly decreased compared to the saline/saline ($p \leq 0.01$) treated group. At Hour 3, the saline/LPS treated group was significantly less than the saline/saline ($p \leq 0.001$), heroin/saline ($p \leq 0.001$), and heroin/LPS ($p \leq 0.001$) treated group. At Hour 4, the saline/LPS treated group was significantly decreased in activity compared to the saline/saline ($p \leq 0.001$), heroin/saline ($p \leq 0.001$), and heroin/LPS ($p \leq 0.01$) treated groups, while the heroin/LPS treated group demonstrated significantly less activity than the saline/saline ($p \leq 0.01$) and the heroin/saline ($p \leq 0.01$) treated groups, but was actually increased in activity compared to the LPS/saline ($p \leq 0.01$) treated group. At Hour 5, the saline/LPS treated group continued to show decreased activity compared to saline/saline ($p \leq 0.001$) and heroin/saline ($p \leq 0.05$) treated groups. At Hour 6, the saline/LPS treated group was significantly lower than the heroin/saline ($p \leq 0.01$) and heroin/LPS ($p \leq 0.01$) treated groups, but only trended towards significance compared to the saline/saline ($p \leq 0.06$) treated groups. At Hour 7, the saline/LPS treated group displayed decreased activity compared to all groups, saline/saline ($p \leq 0.01$), heroin/saline ($p \leq 0.01$), and heroin/LPS ($p \leq 0.05$) treated groups. By Hour 8, the saline/LPS treated group was still lowered compared to saline/saline ($p \leq 0.001$) and heroin/saline ($p \leq 0.001$) treated groups, but did not reach significance compared to heroin/LPS treated groups.

In addition to the time-dependent effects summarized above, there was a significant main effect of LPS [$F(1,43)=100.60$, $p \leq 0.001$) as well as a significant

interaction of heroin * LPS [F(1,43)=34.747, p≤0.001). Posthoc tests indicated that all four groups were significantly different from each other in the measure of activity: saline/saline treated group was significantly higher than heroin/saline (p≤0.001), saline/LPS (p≤0.001), and LPS/heroin (p≤0.001) treated groups; the heroin/saline treated group was significantly higher than the saline/LPS (p≤0.001) and heroin/LPS (p≤0.05) treated group; and the LPS/heroin treated group was significantly increased compared to the saline/LPS treated group (p≤0.01). To summarize, the groups listed in order of greatest amount of activity to the least amount of activity are as follows: saline/saline, heroin/saline, heroin/LPS, and saline/LPS. **Figure 4.1**

RT-PCR

Hippocampus. In the hippocampus, there was a significant effect of LPS on IL-1 β [F(1, 23)=11.923, p≤0.01], MCP-1 [F(1,23)=9.155, p≤0.01], and IκBα [F(1,23)=14.733, p≤0.001], while iNOS trended towards a main effect of LPS but did not reach significance [F(1,23)=4.077, p=0.057]. TNF-α mRNA levels demonstrated a significant main effect of LPS [F(1,23)=16.030, p≤0.001] and an interactive effect of LPS*heroin [F(1,23)=4.702, p≤0.05]. IL-6 mRNA levels demonstrated main effects in both LPS [F(1,23)=17.240, p≤0.001], heroin [F(1,23)=10.572, p≤0.01], and an interactive effect of LPS * heroin [F(1,23)=11.307, p≤0.01]. Posthoc tests revealed that the mRNA levels of TNF-α and IL-6 in the saline/LPS treated group were significantly increased compared to saline/saline (p≤0.01, p≤0.001, respectively), heroin/saline (p≤0.01, p≤0.001), and heroin/LPS (p≤0.05, p≤0.001)

treated groups. MCP-1 and IκBα mRNA were significantly elevated in the saline/LPS treated group compared to saline/saline ($p \leq 0.05$, $p \leq 0.01$, respectively) and heroin/saline treated groups ($p \leq 0.05$, $p \leq 0.01$), but not compared to the heroin/LPS treated group, indicating the inhibition of MCP-1 was not sufficient to significantly distinguish the LPS treated groups from one another based on heroin treatment. **Figure 4.2 and 4.3**

Cortex. In the cortex, there was a significant effect of LPS on TNF-α [$F(1,23)9.517$, $p \leq 0.01$], IL-6 [$F(1,23)=11.910$, $p \leq 0.01$], and IκBα [$F(1,23)=10.471$, $p \leq 0.01$]. There were no significant main effects of heroin nor were there LPS * heroin interactions among any molecules measured. Posthoc tests revealed a significant difference between the heroin/saline treated group and the saline/LPS treated group in TNF-α mRNA expression ($p \leq 0.05$), and the LPS/saline treated group was significantly increased in IL-6 and IκBα mRNA levels compared to both the saline/saline ($p \leq 0.01$, $p \leq 0.05$) and heroin/saline ($p \leq 0.01$, $p \leq 0.05$) treated groups compared to the LPS/saline treated group. **Figure 4.4 and 4.6**

Striatum. In the striatum, there was a main effect of LPS [$F(1,23)=12.592$, $p \leq 0.005$), main effect of heroin [$F(1,23)=6.160$, $p \leq 0.05$), and an interactive effect of LPS * heroin [$F(1,23)=16.011$, $p \leq 0.001$] in only the measure of MCP-1 mRNA. Posthoc tests of MCP-1 mRNA expression revealed that the LPS/saline treated group was increased significantly from the saline/saline treated group ($p \leq 0.001$), heroin/saline treated group ($p \leq 0.05$), and heroin/LPS treated group ($p \leq 0.001$). There was an interactive effect of LPS * heroin on IL-1β mRNA expression

[F(1,23)=4.446, p≤0.05]; posthoc tests revealed that the saline/saline treated group was significantly lower than the LPS/saline treated group (p≤0.05). There were no other significant differences in the striatum. **Figure 4.5 and 4.6**

Discussion

Major findings

LPS treatment increased the proinflammatory mediator expression of TNF-α, IL-1β, MCP-1, IL-6 and IκBα mRNA in the hippocampus, as shown by the saline/LPS treated group compared to both the saline/saline and heroin/saline treated groups. In contrast, for the same comparison, the cortex only showed increased IL-6 and IκBα in response to LPS, and the striatum only showed increased MCP-1. These are the first to examine this selection of proinflammatory mediator mRNA in comparison to each other and in these specific brain regions. The hippocampus showed an increased inflammatory response, as indicated by the number of mediators that were affected by LPS treatment, is consistent with published findings that the hippocampus is a particularly immunoresponsive area in comparison to most other brain regions.

To date there are no published studies examining the effects of opiates such as morphine and heroin on brain proinflammatory mediators. These results are the first to address this issue, and clearly demonstrate that in the hippocampus, some proinflammatory mediators are significantly affected by co-administration of heroin

with LPS. Specifically, TNF-α and IL-6 are decreased with the administration of heroin in LPS-treated rats compared to those that do not receive heroin in addition to the LPS treatment.

A final finding is that, as expected, LPS treatment decreased general activity in rats in a measure of behavioral depression. Heroin by itself decreased activity slightly but significantly, which was due to the immediate effects of heroin in the first two hours post-treatment. The heroin/LPS treated group demonstrated activity levels that totaled between those of saline/LPS treated group and the heroin/saline treated group. Analysis indicated that while the heroin effects in the LPS/heroin group are still present, the effects of LPS in this group were at least partially mitigated.

TNF-α and IL-6 in behavioral depression

Considering the support in the literature for the role of IL-1β in the hippocampus and behavioral depression, it was somewhat surprising that heroin did not exert significant effects on IL-1β mRNA while still inhibiting behavioral depression. However, the role of IL-1β may be subject to a bias based upon availability of knowledge regarding this cytokine, which has been known for much longer than most other proinflammatory cytokine and studied at length. TNF-α and IL-6 mRNA were significantly suppressed in the heroin/LPS treated group compared to the saline/LPS treated group, making either or both of them more likely targets for

the inhibition of behavioral depression also see in the heroin/LPS treated group compared to the saline/LPS treated group. Although TNF-α has been researched extensively, there are few, if any, studies implicating it causally in behavioral depression and intrahippocampal injections of TNF-α do not induce behavioral depression (Pauli et al., 1998). Thus, it is unlikely that TNF-α is the primary cytokine responsible for the alterations seen between the saline/LPS and heroin/LPS treated groups.

The results of these experiments indicate a role of IL-6, which may have been initially underestimated in this behavior. IL-6 is a later-induced cytokine compared to IL-1β, and this experiment was designed to examine later timepoints (Laye et al., 1994). It may be that with the extended time (8 hours post-treatment) used in these experiments, IL-6 was a more integral part of the pathway leading to behavioral depression, and reversal of IL-6 production by heroin led to an amelioration of behavioral depression. Given the significant overlap between behavioral depression and learning, it is worth consideration that IL-6 deficient mice were resistant to LPS-induced impairments in the Morris water maze, a spatial task dependent upon the hippocampus, and had reduced IL-1β and TNF-α levels in the brain while retaining normal levels in peripheral plasma (Sparkman et al., 2006). This study is intriguing in light of the results presented above, suggesting IL-6 is necessary for the brain to produce or sustain IL-1β and TNF-α and that this may account for reversal of hippocampal impairments. However, it is important to note that the results in this dissertation do not address learning and memory impairments specifically, as well as the fact that IL-1β levels were not suppressed in the heroin/LPS treated animals as

one might expect if a lack of IL-6 were preventing IL-1β production, although IL-1β levels were not significantly elevated by LPS regardless. A more closely related published experiment indicated that IL-6 deficient mice did not exhibit reduced social activity, a facet of behavioral depression, when given LPS, compared to their wild type counterparts (Mingam et al., 2008). Likewise, IL-1 deficient mice also displayed attenuation of reduced social exploration, body weight and immobility to LPS (administered either systemically or intracerebroventricularly) (Bluthe, Michaud, Poli, & Dantzer, 2000). Anti-serum to IL-6 also inhibits behavioral depression after LPS treatment, while anit-sera to TNF-α and lL-1β had no such effect (Harden et al., 2006). Collectively, these previous studies suggest that production of IL-6 in the hippocampus is an integral part of the inflammatory response carried out in that brain region, and the results from this study support this hypothesis by demonstrating that the lack of LPS-induced IL-6 mRNA in the hippocampus is correlated with a reduction of behavioral depression induced by LPS.

Intermediates of cytokines and behavior

While it is apparent that proinflammatory cytokines such as IL-1β produce deficits in hippocampally-dependent learning, the cytokines themselves cannot directly induce behavioral changes. One potential intermediate between cytokines and behavior is PGE_2. Inhibition of COX-2 reverses the learning impairments caused by IL-1β, indicating a potential role for PGE_2 in the hippocampal disruptions (Hein et al., 2007). However, the role of PGE_2 in the hippocampus is not nearly as

established as it is in the hypothalamus, and it is possible that proinflammatory mediators can bypass this step. Interestingly, behavioral depression has also been investigated as a result of glycoprotein 120, an envelope protein on the human immunodeficiency virus (HIV) that is believed to be one of the main inflammatory agents of HIV. Intracerebrally injected gp120 induces a number of sickness behaviors, including behavioral depression and fever, which were associated with increased IL-1β and PGE_2 in both hippocampus and hypothalamus. However, neither prevention of PGE_2 synthesis and antagonizing IL-1β centrally affected the behavioral depression seen with gp120, although it did prevent the fever response (Barak et al., 2002a; Barak et al., 2002b). These experiments suggest that although cytokines and prostaglandins are involved in the process of producing behavioral depression, there are other variables such as other cytokines like IL-6 that can take over the process.

Clearly, neurons are an important part of this process, and specifically, serotonergic transmission is vital to the behavioral effects of proinflammatory mediators in the hippocampus, although there is some evidence for norepinephrine also being involved in these processes. Interestingly, serotonin is a key neurotransmitter in controlling activity via projections to many motor areas of the brain (Jacobs & Fornal, 1995). Measurement of serotonin via microdialysis in the hippocampus *in vivo* has shown that LPS causes increased hippocampal serotonin release as well as norepinephrine to a lesser degree (Lacosta, Merali, & Anisman, 1999; Linthorst et al., 1998). Systemic IL-1β and IL-6 both increase serotonin activity in the hippocampus (Zalcman et al., 1994). Minocycline, a microglial

inhibitor, reduced IL-1β, IL-6, and indoleamine 2,3 dioxygenase (commonly used measure for serotonin activity) in the hippocampus after LPS treatment and also suppressed sickness behavior, which indicates the cytokines and serotonin are part of the pathway involved in sickness behavior elicited from the hippocampus (Henry et al., 2008). Importantly, serotonin increases in healthy, active rats and decreases during rest and sleeping; however, when an immune challenge is present, serotonin release is increased while activity is decreased (Linthorst et al., 1994). Serotonin receptors are also increased in the hippocampus after chronic administration of IL-1β (Anisman et al., 2008). Interestingly, IL-6 is implicated in increasing serotonin turnover in the hippocampus in a non- PGE_2 dependent manner (Wang & Dunn, 1998; Barkhudaryan & Dunn, 1999). This supports our findings that IL-6 levels inversely correlate with activity, while COX-2, an indicator of PGE_2 activity, does not. Serotonin dose-dependently suppresses LTP in hippocampal slices, providing a potential mechanism through which proinflammatory mediators can impair LTP, as noted above, and thus impair learning ability (Staubli & Otaky, 1994; Hauss-Wegrzyniak et al., 2002). While these experiments do not specifically address serotonin, given the evidence available, it seems likely that heroin, by suppression of IL-6 and TNF-α, would also suppress serotonin, resulting in the alleviation of behavioral depression that was clearly measured in the experiments above.

Conclusion

The experiments completed in this chapter are the first to utilize RT-PCR to comprehensively examine proinflammatory mediators in hippocampal-specific

responses to LPS stimulation. Moreover, these are the very first studies to begin to inspect the relationship between opiates and proinflammatory mediators in the brain and subsequent sickness behaviors. In addition to the many risk factors opiates users are known to have in terms of infections, these data indicate that opiates may also prevent adaptive sickness behaviors as a result of altered proinflammatory mediator responses in the brain. Although there are important implications for heroin induced suppression of IL-6 and TNF-α in the hippocampus, it may prove that the use of heroin to suppress these events is a useful tool for understanding the complex pathways involved in neuroimmune communication and sickness behaviors, which are discussed further in the next chapter.

Table 3. Significance values by group for the duration of the 8-hour experiment measuring general activity after 1mg/kg heroin or saline and 1mg/kg LPS or saline treatment.

		Baseline	Hour 1	Hour 2	Hour 3	Hour 4
saline/saline						
	heroin/saline	0.967	0.001	0.001	0.696	0.963
	saline/LPS	0.914	1.000	0.003	0.001	0.001
	heroin/LPS	0.878	0.001	0.001	0.833	0.002
heroin/saline						
	saline/saline	0.967	0.001	0.001	0.696	0.963
	saline/LPS	0.667	0.001	0.703	0.001	0.001
	heroin/LPS	0.608	0.993	0.967	0.994	0.007
saline/LPS						
	saline/saline	0.914	1.000	0.003	0.001	0.001
	heroin/saline	0.667	0.001	0.703	0.001	0.001
	heroin/LPS	1.000	0.001	0.421	0.001	0.005
heroin/LPS						
	saline/saline	0.878	0.001	0.001	0.833	0.002
	heroin/saline	0.608	0.993	0.967	0.994	0.007
	saline/LPS	1.000	0.001	0.421	0.001	0.005
		Hour 5	**Hour 6**	**Hour 7**	**Hour 8**	
saline/saline						
	heroin/saline	0.116	0.773	0.999	0.328	
	saline/LPS	0.001	0.065	0.002	0.001	
	heroin/LPS	0.014	0.832	0.887	0.001	
heroin/saline						
	saline/saline	0.116	0.773	0.999	0.328	
	saline/LPS	0.029	0.004	0.003	0.001	
	heroin/LPS	0.808	0.999	0.929	0.001	
saline/LPS						
	saline/saline	0.001	0.065	0.002	0.001	
	heroin/saline	0.029	0.004	0.003	0.001	
	heroin/LPS	0.202	0.006	0.014	0.139	
heroin/LPS						
	saline/saline	0.014	0.832	0.887	0.001	
	heroin/saline	0.808	0.999	0.929	0.001	
	saline/LPS	0.202	0.006	0.014	0.139	

Figure 4.1 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means by treatment group with standard error of means (SEM). Activity was measured by biotelemetry devices to indicate gross motor movement. * denotes significance of at least p≤0.05. **(a)** Activity counts were summed over each hour for each rat and analyzed per treatment group over the 8 hours after treatment. **(b)** Activity counts were summed over the entire period of 8 hours post-treatment and compared to indicate treatment differences regardless of time.

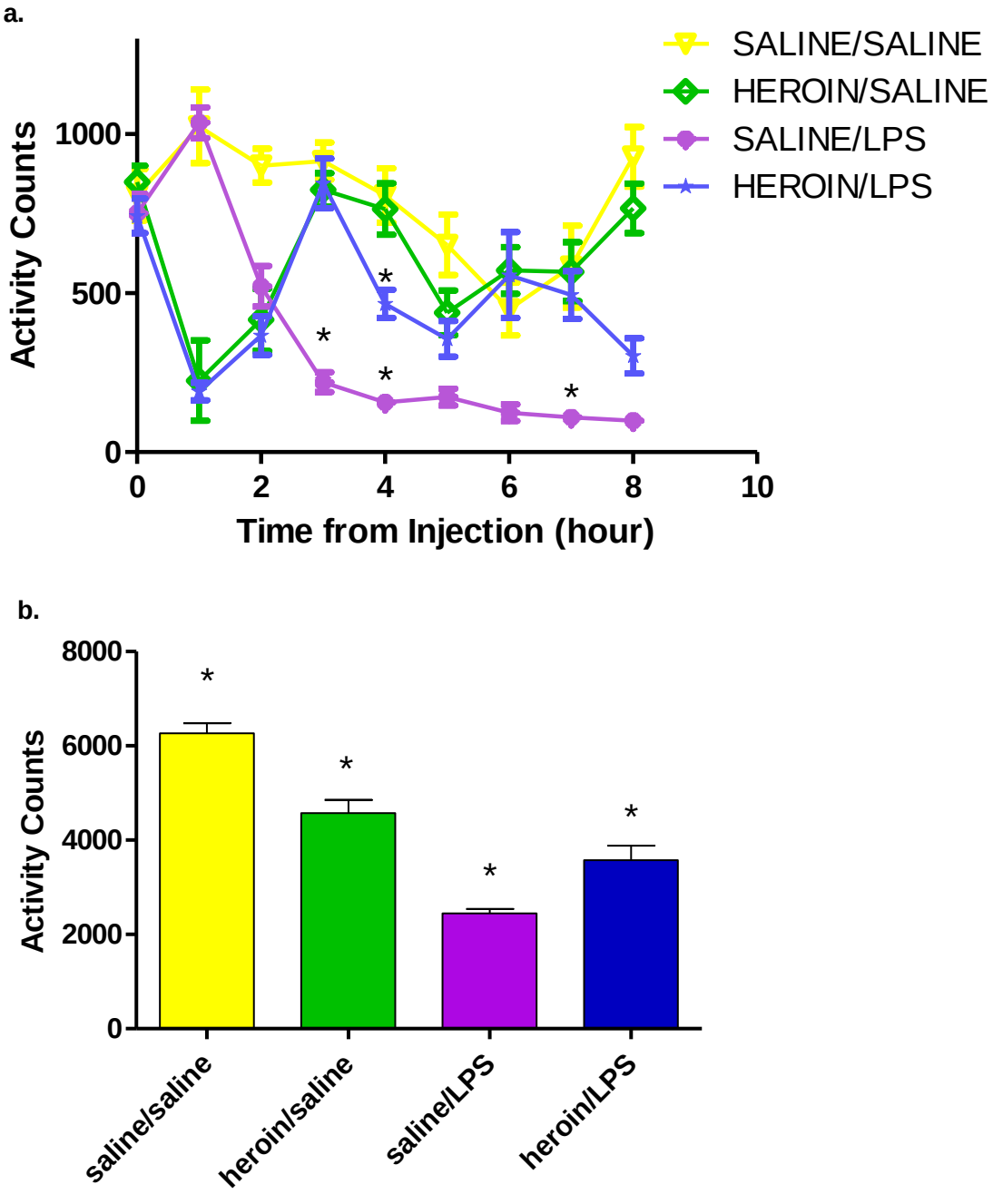


Figure 4.2 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels measured in the hippocampus. **(a)** TNF-α **(b)** IL-1β **(c)** iNOS **(d)** IL-6 **(e)** MCP-1

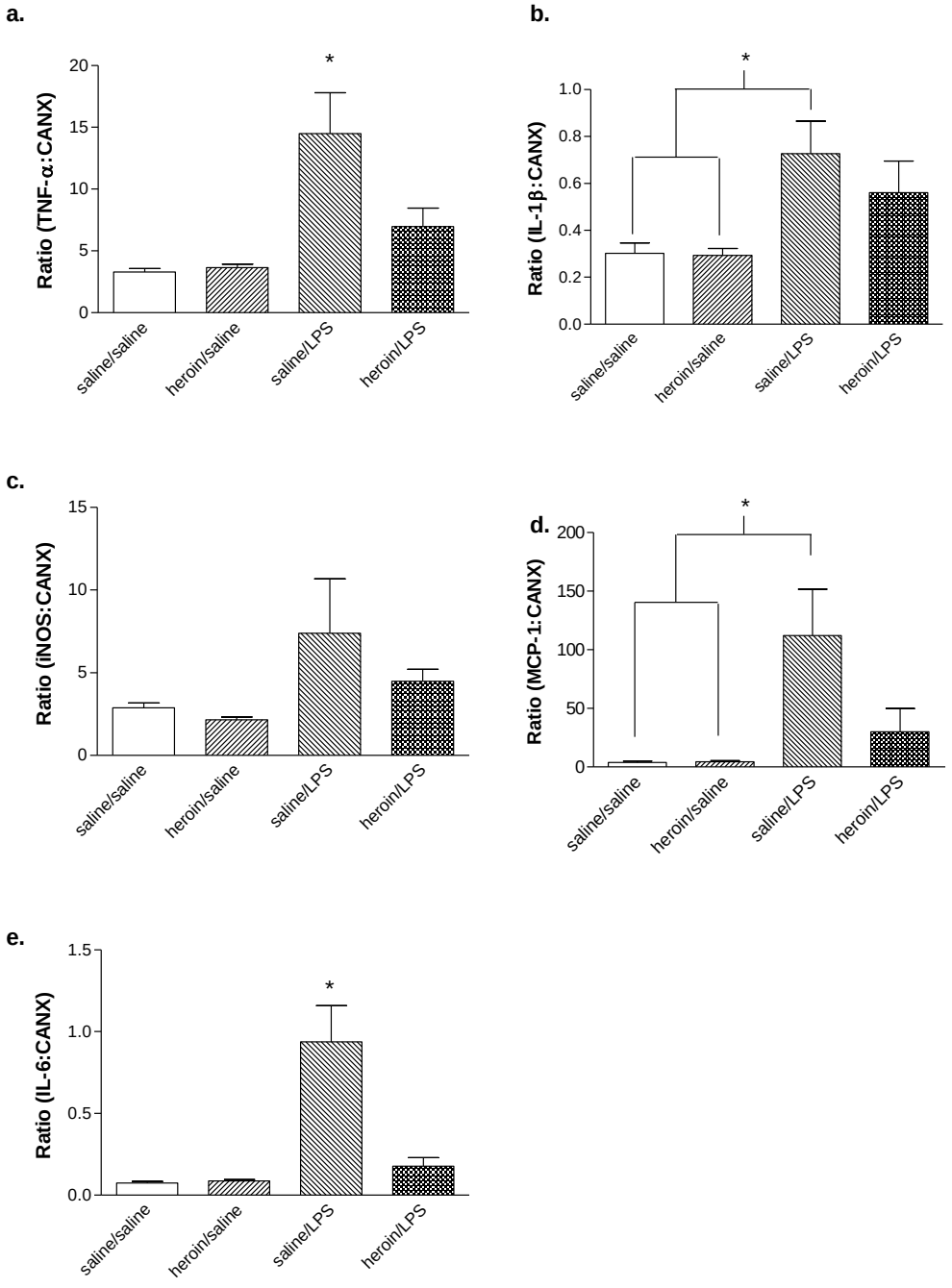
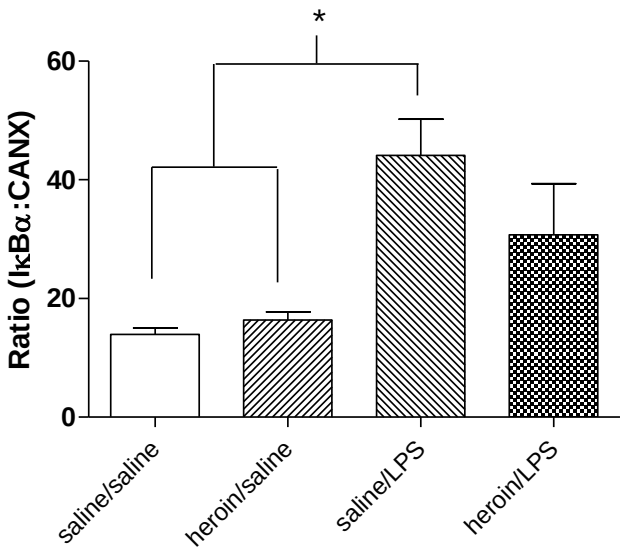


Figure 4.3 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graph represents the mRNA levels of (a) IκBα and (b) COX-2 measured in the hippocampus.

a.



b.

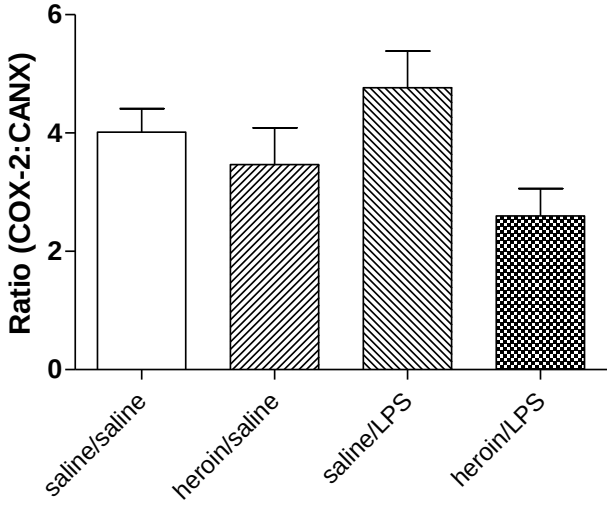


Figure 4.4 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels measured in the cortex. **(a)** TNF-α **(b)** IL-1β **(c)** iNOS **(d)** IL-6 **(e)** MCP-1

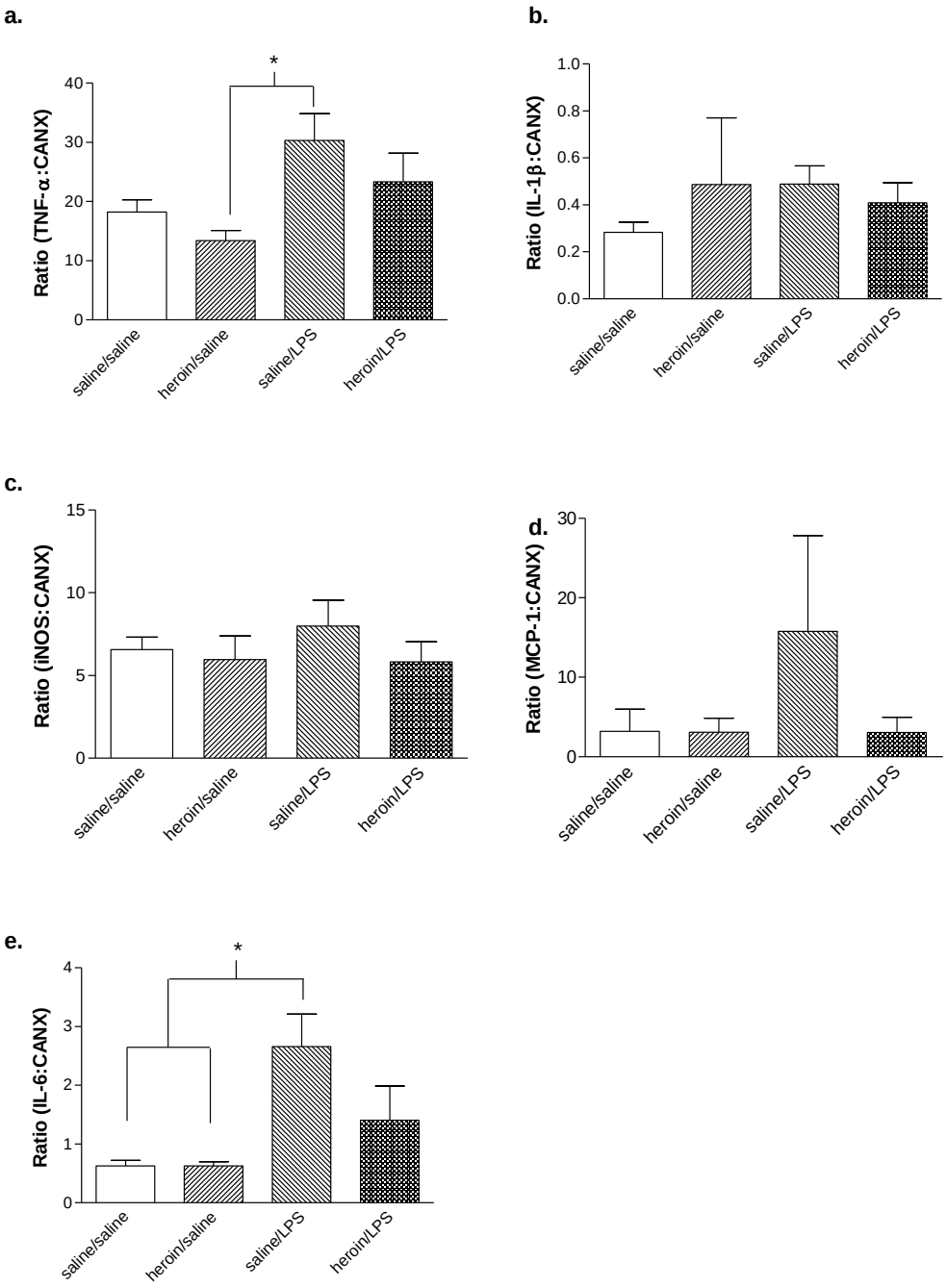


Figure 4.5 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels measured in the striatum. **(a)** TNF-α **(b)** IL-1β **(c)** iNOS **(d)** IL-6 **(e)** MCP-1

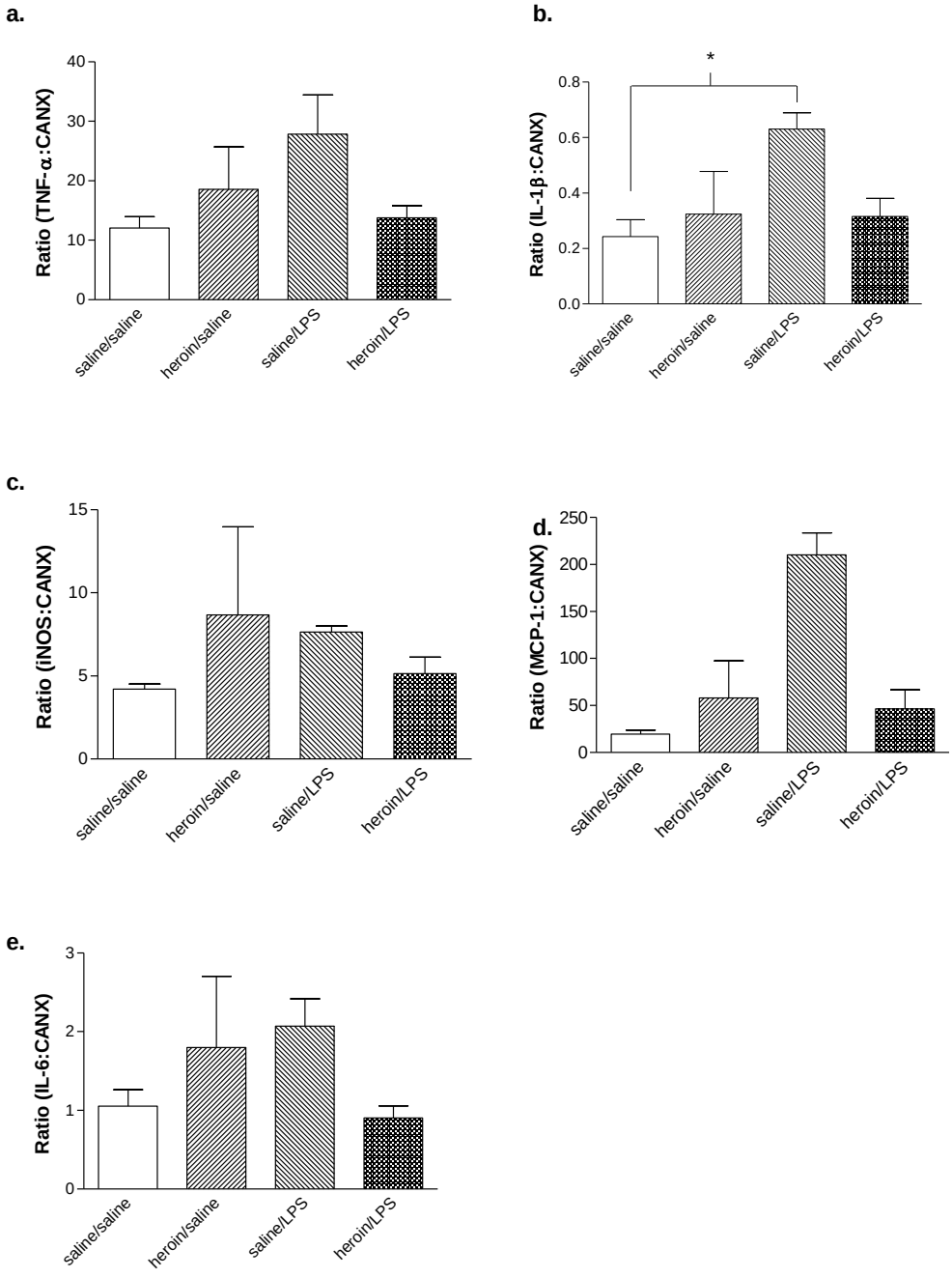
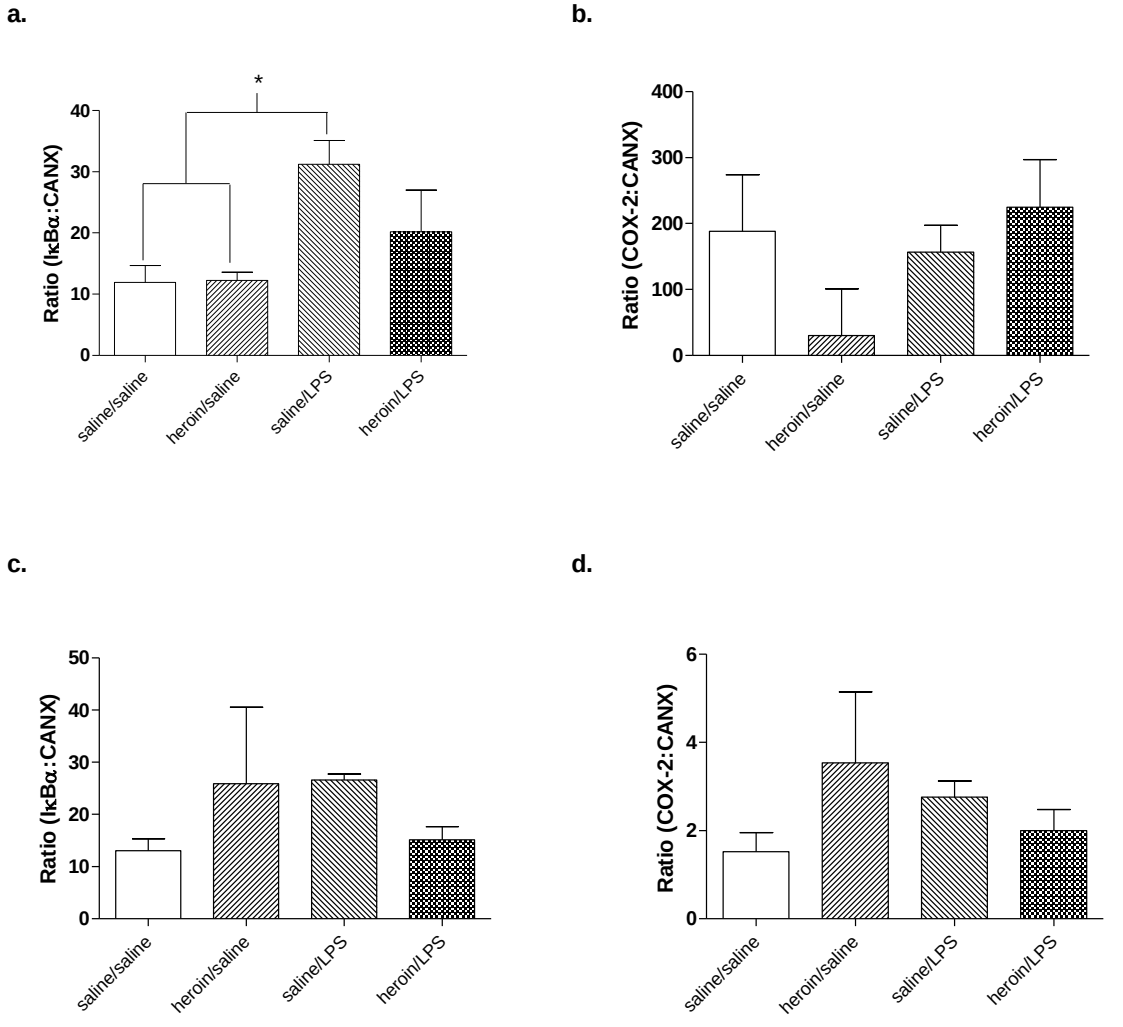


Figure 4.6 Rats were treated with heroin (1mg/kg, s.c.) or saline and LPS (1mg/kg) or saline. Sacrifice occurred 8 hours after treatment. Data is represented as means and SEM of mRNA ratios compared to housekeeping gene CANX as measured through RT-PCR. * denotes significance of at least p≤0.05. The following graphs represent the mRNA levels of **(a)** IκBα and **(b)** COX-2 measured in the cortex, and the mRNA levels of **(c)** IκBα and **(d)** COX-2 in the striatum.



Chapter 5

General Discussion

Primary Findings

The present series of experiments examined production of proinflammatory mediators in the brain as a result of heroin and/or LPS treatment as well as behavioral measures that correlate with brain proinflammatory mediators. Previously published work has clearly established that proinflammatory mediators are important to the production of sickness behaviors, particularly fever; however, none have addressed the relationship between opiates and brain proinflammatory mediators.

Chapter 2 examined the potential mechanism of heroin-induced hyperthermia, and concluded that this hyperthermic effect of heroin does not recruit the traditional fever pathway. However, heroin does produce a marked increase in body temperature, despite the independence from increased proinflammatory mediators and insensitivity to the PGE_2 synthesis inhibitor, indomethacin.

Chapter 3 presented studies that examined the effect of heroin on fever production and molecules involved in the traditional fever pathway. These results are the first to examine a wide array of mRNA of LPS-induced proinflammatory mediators in the hypothalamus during fever, and the very first to address the effects

of opiates on hypothalamic production of proinflammatory mediators during fever as a potential mechanism for attenuation of fever. Additionally, it was established that fever induced by LPS was impaired when heroin was also administered, presumably via the suppression of proinflammatory mediators by heroin.

Chapter 4 consisted of experiments demonstrating that like fever, heroin also inhibited production of proinflammatory mediators in the brain and this was consistent with the impairment of behavioral depression. The hippocampus is not as well-established as the hypothalamus as an immunologically active region of the brain, although multiple studies have shown its involvement in directing behavioral depression in the event of an immune challenge. These results contribute to those findings by demonstrating that LPS strongly induces proinflammatory mediators in this region, and heroin has an inhibitory effect on some of those proinflammatory mediators. Interestingly, while the hypothalamus shows a robust effects in all measured proinflammatory mediators, the hippocampus shows a far more selective response which remains to be elucidated.

Potential consequences of heroin-induced hyperthermia

While fever is an adaptive response to an immune challenge, increased body temperature overall is not likely to be beneficial, and is more likely to be detrimental to the health of the organism due to the high metabolic cost of sustaining a raised body temperature. Hyperthermia (fever or not) enhances the ability of many immune cells to perform vital defense functions (Manzella et al., 1979; Sebag et al., 1977). However, when an immune challenge is not present, the cost of optimization of

these cells is unlikely to be a favorable outcome. Overall, the ability of heroin to produce hyperthermia would impose a requirement of energy for little to no benefit to the organism.

There are a number of potential avenues of research to follow up on these findings. First, there is the obvious question of mechanisms. Many studies have already shown that the μ receptor is involved in opiate-induced hyperthermia and that this could potentially alter thermosensitive neuron sensitivity. However, it would be interesting to determine exactly how this works- do the μ receptors reside on thermosensitive neurons directly, or do they require immune cells in some manner? What is the natural role of endogenous opioids such as β-endorphin in production of fever? These are questions that could be answered regarding the hyperthermia.

A more intriguing avenue of research, in this author's opinion, would be to determine how heroin-induced hyperthermia affects immune regulation in the rest of the body. As noted above, hyperthermia has been shown to affect immune cell function. However, these studies were done *in vitro*, which limits the application of their results. *In vivo* determination of whether or not immune cells are affected by heroin-induced hyperthermia would help determine the downstream effects of this phenomenon. Much of the support for fever as a beneficial response to microbial infection comes from disease outcome studies. Since heroin-induced hyperthermia is not an infection-induced reaction, it would be interesting to see how the hyperthermia might affect non-disease outcomes more relevant to every day living as opposed to special circumstances (such as infection), particularly those associated with hypothalamic functions and homeostasis.

Heroin-induced hyperthermia and activity

Hyperthermia that is not fever is seen in a number of conditions, including neuroleptic malignant syndrome and sympathomimetic poisoning by cocaine or amphetamines. All of these conditions involve the hypothalamus, but are not responsive to prostaglandin inhibition (Stitt, 1979). These known hyperthermias can shed some light onto opiate-induced hyperthermia. One line of thought is that heroin-induced hyperthermia is related to an increase in activity, similar to that seen in sympathomimetic poisoning as a result of overactivation of the sympathetic nervous system by cocaine or amphetamines (Halloran & Bernard, 2004; Rusyniak & Sprague, 2005). Opiates do cause an increase in activity in mice (Yoo et al., 2003); however, heroin in rats actually produces a substantial decrease in activity during the same time period that hyperthermia occurs (see Figure 4.1 and Table 3). Neuroleptic malignant syndrome occurs when an individual is taking drugs that block dopamine receptors, or when dopaminergic agents are suddenly withdrawn from the patient (usually Parkinson's patients), resulting in muscle rigidity and hyperthermia that can be treated with dopamine agonists (Halloran et al., 2004; Newman, Grosset, & Kennedy, 2009; Rusyniak et al., 2005). This indicates that an imbalance in dopamine results in hyperthermia when the imbalance is tilted in the direction of too little dopamine. What we know about sympathomimetic poisoning and neuroleptic malignant syndrome indicates that heroin-induced hyperthermia is distinct from these conditions, as heroin reduces activity (differs from sympathomimetic poisoning) and increases dopamine (differs from neuroleptic malignant syndrome).

Like hyperthermia, a lack of activity is reminiscent of sickness behaviors, yet does not seem to be a result of immune activation as seen by the lack of proinflammatory mediator mRNA in the hippocampus 90 minutes after heroin administration. Also like hyperthermia, a decrease in activity is unlikely to be beneficial to the organism when not facing an immune challenge, and may represent a shift in motivation as seen in sickness behaviors that, instead of being advantageous, will cause behaviors that are inappropriate for the current environment. The mimicry of sickness behaviors by heroin is unlikely to be adaptive when no immune challenge is present, and Chapters 3 and 4 address the potential problems that heroin presents in sickness behaviors when an immune challenge is on board.

Heroin immunosuppression in the hypothalamus

This is the first study to comprehensively examine proinflammatory mediators via RT-PCR in the hypothalamus as a result of systemic LPS administration, and the results are consistent with other studies suggesting the roles of the proinflammatory mediators suggested. The effect of opiates on the proinflammatory mediators in the brain, however, is entirely original. These very novel findings demonstrated that proinflammatory mediators are affected and likely involved in the suppression of fever; however, the experiments described in this dissertation are focused on establishing the effect, not addressing the mechanisms. The logical next step, then, would be to determine those mechanisms through which opiates can suppress proinflammatory mediators.

Communication between peripheral immune activation and hypothalamus

Heroin, as a μ agonist, could affect the immune response in a number of ways. To understand the points in which heroin might affect, one must first consider the pathways of normal immune-neural communication. There are many hypotheses regarding the communication that must occur for the immune system to signal to the brain that an immune challenge has occurred. Although it is possible that cytokines from the bloodstream can signal to produce more cytokines in the brain, particularly in the hypothalamus, cytokines are relatively large molecules that cannot easily cross the blood brain barrier (Watkins, Maier, & Goehler, 1995). There could also be specialized uptake mechanisms to aid cytokines in crossing the blood brain barrier, but these have not been found, although macrophage infiltration is also a possibility (Dunn, 2006). Interestingly, the most prominent theory involves the vagal nerve and norepinephrine, although this does not exclude the possibility of other pathways also playing a role in peripheral immune communication to the brain (Maier et al., 1998a; Hansen et al., 2000). Some of the most apparent changes that occur in the brain due to autonomic input during an immune reponse take place in the hypothalamus and the hippocampus, which is consistent with the regions implicated in producing sickness behaviors (Carlson, Felten, Livnat, & Felten, 1987).

The vagus nerve contains afferent projections to the brain from the peripheral organs via the nucleus of the solitary tract (NTS), which then projects to the locus coeruleus (LC) (Van Bockstaele, Peoples, & Telegan, 1999). Specifically regarding

proinflammatory mediators and sickness behaviors, vagotomy prevents fever from peripherally administered IL-1β, but fever still occurs if PGE_2 is introduced directly into the brain despite vagotomy (Milligan et al., 1997; Watkins et al., 1995). The LC projects to many areas of the brain, most notably to the hypothalamus but also to the hippocampus, and has been implicated in providing the innervation with norepinephrine release in the hypothalamus (Dunn, 2006). Peripheral stimulation of this pathway by proinflammatory cytokines causes a release of norepinephrine, and the norepinephrine projections to the hypothalamus, in turn, cause an increase in proinflammatory cytokine production there (Blandino, Jr., Barnum, & Deak, 2006; Zalcman et al., 1994). Peripheral LPS treatment stimulates release of norepinephrine in the preoptic area of the hypothalamus, as confirmed by microdialysis studies (Lavicky & Dunn, 1995; Linthorst et al., 1998). Proinflammatory cytokine are produced in the brain by microglia, as indicated by the ability of the microglia inhibitor minocycline to decrease stress-induced IL-1β in the hypothalamus but not spleen (Blandino, Jr. et al., 2006). These are the events that make up the most likely pathway of normal immune activation and fever production by the hypothalamus during an immune challenge.

Potential mechanisms of opiates on immune-neural communication to the hypothalamus

Opiates have the potential to alter almost every step of this pathway from the initial signals to the vagus nerve to proinflammatory cytokine production in the hypothalamus. Cultured monocytes (cells from which macrophages and microglia

are derived) treated with morphine have a suppressed TNF-α and IL-6 response to immune challenge, which is mediated by μ receptors (Bonnet, Beloeil, Benhamou, Mazoit, & Asehnoune, 2008). This effect is likely due to μ receptors that are present on macrophages (Chuang et al., 1995). These mechanisms could have an effect on the initiation events to signal to the hypothalamus; that is, the vagus nerve would receive no or a diminished signal and subsequent pathways would respond (or not) accordingly.

While it is possible that opiates can inhibit immune responses by acting directly on peripheral immune cells via μ receptors, it is unlikely that this can account for all of the effects of opiates. One study indicated that μ antagonists administered directly into the brain increased serum IL-6 (Bertolucci, Perego, & De Simoni, 1997). This indicates that the μ receptor plays an integral role in altering cytokine levels (albeit peripheral cytokine levels) via a central mechanism. One might infer that μ agonists would have the opposite effect and decrease IL-6 in the serum, and this finding could potentially extend to the central cytokines as well. Unfortunately, while it is common for investigators to measure peripheral cytokines, brain cytokines and other proinflammatory mediators have not been examined in respect to effects of μ receptor influence. Using the knowledge presented above, we must then speculate on how central μ receptors may influence brain proinflammatory mediator production.

After the immune cells in the periphery signal the vagus nerve, μ receptors may then play another role through the vagal nerve. There are μ receptors present on vagal afferents that project to the NTS, which seem to influence cardiopulmonary

and gastrointestinal responses, resulting in a reduction in excitatory postsynaptic potentials through a calcium dependent mechanism (Atweh, Murrin, & Kuhar, 1978; Aicher, Goldberg, Sharma, & Pickel, 2000; Poole, Deuchars, Lewis, & Deuchars, 2007; Rhim & Miller, 1994). A reduction in excitation of the vagal nerve could also have the downstream effect of altering brain immune responses and sickness behaviors.

The final brain region involved in this proposed signaling pathway before reaching the hypothalamus is the locus coeruleus (LC), which could be affected by μ receptor activation through at least two potential mechanisms. This region is well known for being the primary source of norepinephrine for the brain, as well as being intimately involved in the effects opiate withdrawal. Acute opiates have an inhibitory effect on the LC, which is associated with μ receptors that are coupled with G protein-coupled inwardly-rectifying potassium (GIRK) channels (Christie, 1991; Torrecilla et al., 2002). In addition to the μ receptors located within the LC, the periaqueductal gray (PAG), a region associated with opioid-induced analgesic effects and a large population of μ receptors, has inhibitory afferents to the LC, which could also inhibit the excitation of this region necessary for transmission of the autonomic signals received during an immune challenge (Aston-Jones et al., 1991). However, the action of the PAG has been linked only to suppression of natural killer cell activity in the spleen by morphine, which indicates this region may influence efferents from the central nervous system to the periphery as opposed to the afferents coming in from the periphery to the central nervous system that influence

production of proinflammatory mediators within the brain (Carr, Gebhardt, & Paul, 1993).

There are many potential ways that heroin could influence brain proinflammatory mediators in the hypothalamus and subsequent fever production. Since this is the first time that the suppressive effect of heroin (or any opiate) on brain proinflammatory mediators has been shown in this manner, there are many possibilities for potential mechanisms. Due to the influence of the hypothalamus on peripheral mechanisms, understanding brain proinflammatory activity may be of use to determine peripheral responses that are controlled by the central nervous system. Additionally, the "normal" pathways for immune activation by infection or other immune challenge is still controversial. There is still much to be done in this area of interest.

Connections between serotonin, hippocampus, activity, and immune activation

Unlike the hypothalamus and its role in fever, the role of the hippocampus in regulation of activity is not as commonly known amongst neuroscientists, and the interactions with proinflammatory mediators is even less known. The pathway from peripheral inputs to the hippocampus is likely to be similar to that of the hypothalamus until the point of the NTS, and possibly the LC. The connections to the hippocampus are too numerous and complex to enumerate here, although some discussion is necessary in light of the results found in this dissertation. Although

very little research has been in regards to brain proinflammatory mediators and the hippocampus, we can learn about potential neurocircuitry involved by examining established connections from non-sickness behavior oriented perspectives.

Although the hippocampus is typically regarded as having a “learning and memory” function in neurobiology, this does not exclude the potential for other roles of this important brain region. In particular, the serotonergic system and its connections in the hippocampus have been linked to the reduction in activity that is seen as a component of sickness behaviors. Although often overlooked due to its many roles in other behaviors, the serotonin system is intricately involved in regulation of large muscle movements (as opposed to small, fine movements) (Jacobs et al., 1995). Electrical activity in the hippocampus correlates strongly with gross movement such as walking and jumping, but not with small movements, such as licking or eating (Bland et al., 1972; Vanderwolf, 1969). It has been proposed that the hippocampus, through serotonin, regulates gross motor movement, and the removal of the hippocampal control results in a dysregulation of movement control (Vanderwolf, 2001). Disruption of electrical rhythms in the hippocampus causes a complete halt of those gross motor movements, and lesions of the hippocampus cause hyperactivity at first, and then hypoactivity, which is believed to be a result of an inability to control the “lower” motor regions (Vanderwolf, 2001; Vanderwolf, McLauchlin, Dringenberg, & Baker, 1997). Under normal, non-immune activated conditions, serotonin positively correlates with activity (Linthorst et al., 1994; Jacobs et al., 1995).

During an immune challenge, however, activity decreases substantially while serotonin increases significantly (Linthorst et al., 1998; Linthorst et al., 1994). Immune challenge, then, appears to cause contradictory actions in the hippocampus regarding serotonin. Increased serotonin is seen with peripheral LPS as well as with intrahippocampal IL-1β or LPS (Linthorst et al., 1998; Linthorst et al., 1994). Systemic IL-1β and IL-6 both cause increased serotonin in the hippocampus (Zalcman et al., 1994). This is not to say that other neurotransmitters are not involved when an immune challenge as there are also moderate increases in norepinephrine, although not comparative to the increases in serotonin in the hippocampus or the increases of norepinephrine seen in the hypothalamus. Dopamine has also been shown to be increased as in some regions such as the prefrontal cortex, but is associated more with a generalized stress response than a specific immune response (Zalcman et al., 1994; Dunn, Wang, & Ando, 1999).

Not only is serotonin increased with immune challenge, but proinflammatory mediators appear to increase serotonin. *In vitro* studies have shown that the addition of a serotonin antagonist to lymphocyte and macrophage cultures causes a substantial decrease in IL-6 and TNF-α production (Kubera, Maes, Kenis, Kim, & Lason, 2005). This is particularly interesting given that the results presented here in Chapter 4 indicated that IL-6 and TNF-α were the only two proinflammatory mediators in the hippocampus to be significantly increased with LPS/saline treatment and then decreased significantly in the heroin/LPS treated group. As noted before, it was somewhat surprising that IL-1β did not show these effects, but the literature supports the idea that IL-6 might be more involved in hippocampal and

subsequent activity behavior, while IL-1β retains its well-supported role in hypothalamic actions and fever production. This is corroborated by studies that show that IL-1β affects norepinephrine turnover and activation of hypothalamus, while IL-6, both peripheral and centrally administered, did not significantly increase norepinephrine turnover but did increase serotonin turnover in the brain, including the hippocampus (Wang et al., 1998; Zalcman et al., 1994). IL-1β deficient mice also show impaired fever response to LPS, but still displayed a reduction in activity, indicating that IL-1β is vital to fever production but not may be as important to activity reduction during an immune challenge (Kozak et al., 1995). Consistent with our results, IL-6 increases serotonin but does not necessitate PGE_2 in order for effects mediated by serotonin to occur (Barkhudaryan et al., 1999).

Potential mechanisms of opiates on immune-neural communication to the hippocampus

Norepinephrine is most implicated in hypothalamic activation and fever production, but serotonin is the neurotransmitter most likely to be primarily involved in the proinflammatory response in the hippocampus and subsequent behavioral depression. Most serotonin in the brain originates from the raphe nuclei, which have a number of projections to other brain regions. Specifically, the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) both contain primarily serotonergic neurons, but differ substantially in their projections and effects. Both of these regions affect serotonin in the hippocampus, and may play differential roles in the motor regulation by the hippocampus and serotonergic transmission. The MRN in

particular has been shown to be largely responsible for serotonin projections to the hippocampus, although some studies indicate that the MRN is largely involved in dorsal hippocampal function while the DRN is more involved in ventral hippocampal function (Coplan & Lydiard, 1998; Azmitia & Segal, 1978). The output from the hippocampus to affect activity may include a number of brain regions, including the motor cortices, but also the subthalamic nucleus and posterior hypothalamus (Jacobs et al., 1995; Bland & Vanderwolf, 1972; Vanderwolf, 2001).

Unfortunately, since the hippocampal connections are so complex and little attention has been given to these connections in conjunction with opioid receptors and effects of opiates, it is difficult to speculate as much on potential mechanisms through which heroin might exert the immunosuppressive effects seen here on IL-6 and TNF-α in the hippocampus. The LC, as mentioned before, has a significant number of μ receptors, and also has noradrenergic projections to the hippocampus (Coplan et al., 1998). However, given the relationship between serotonin and proinflammatory mediators in the hippocampus, particularly IL-6, it is less likely that noradrenergic projections are responsible for heroin's immunosuppressive effects instead of serotonin. The DRN, which is adjacent to the PAG, has inhibitory μ receptors on both GABAergic and glutamatergic neurons, which indicates that binding of these μ receptors could increase or decrease serotonin release from this area (Aghajanian & Sanders-Bush, 2002; Jolas & Aghajanian, 1997). Additionally, μ receptors have been found in both the amygdala, which has projections to the hippocampus, and in the hippocampus itself (Mansour, Fox, Burke, Akil, & Watson, 1995; Mansour, Fox, Thompson, Akil, & Watson, 1994).

In short, due to the complexity of projections to and from the hippocampus in addition to the lack of research in this area specifically concerning sickness behavior, any suggestions of the pathways involved are preliminary at best. Certainly, there is a great deal of research potential in this area to determine the neuroimmune interactions involving the hippocampus that remains to be completed.

Future directions and limitations of the current findings

The findings presented in this dissertation determined that heroin produces hyperthermia while not altering proinflammatory mediators by themselves, but when in the presence of an immune challenge (LPS), heroin has a suppressive effect on proinflammatory mediators in the hippocampus and hypothalamus in the brain, and thus also suppress associated sickness behaviors- behavioral depression and fever, respectively. While I have proposed potential pathways for these interactions in the General Discussion, experiments in this dissertation did not aim to determine the pathways through which opiates may exert these effects. Experiments designed to address potential pathways for neuroimmune interactions with opiates will, hopefully, be done in the future.

There are a few limitations of the experiments presented in this dissertation that should be acknowledged. The experiments were designed specifically to measure mRNA of proinflammatory mediators. The only protein measured in these studies was β-endorphin, which was done because β-endorphin is cleaved from POMC, and the transcription of POMC does not necessarily indicate that β-endorphin levels as a number of other proteins are also derived from POMC. The

measurement of mRNA of proinflammatory mediators is both a strength and limitation. By using mRNA as the measure, I have established that cells are in the process of producing these proinflammatory mediators. While our RNA extraction method allows for site-specific (i.e., hypothalamus versus hippocampus) extractions, it does not allow us to determine the cellular origin of the proinflammatory mediator mRNA (i.e., microglia versus neuron). Additionally, not everything that is being upregulated in mRNA in the cell necessarily makes it to fully-formed protein, nor does it necessarily get secreted by the cells. This could be remedied by also measuring protein. Unfortunately, due to the low amounts of protein in brain tissue, it would be difficult if not impossible to measure protein and mRNA from the same animals. To confirm that protein is produced and biologically active, a number of assays could be used, including Western blot or enzyme linked immunosorbent assays (ELISAs), although this would require an entirely new series of experiments independent of mRNA results. However, unlike the mRNA results, protein results cannot determine where the proinflammatory mediators originated- for example, protein could indicate that proinflammatory mediators are increased, but it cannot determine if those mediators were produced from cells in the hypothalamus or if the proteins were synthesized in the periphery and crossed the blood brain barrier.

Another limitation that should be mentioned is that these experiments are observations of proinflammatory mediators during LPS and/or heroin treatment as opposed to the manipulation of a specific molecule. For instance, a follow up study would be ideal to determine my proposed role of IL-6 in the hippocampus as it relates to a reduction in activity. One potential follow up to the studies presented

here would be to inject IL-6 antibodies into the hippocampus after LPS administration to see if the reduction in activity could be blocked in this manner. This kind of thinking could be carried out on various proinflammatory mediators in both activity and fever behaviors in the hippocampus and the hypothalamus, although some of this work has been done previously in the hypothalamus with mixed results. One must be cautious regarding injections involving cannulations into the brain due to inflammatory reactions that typically surround the cannula for man weeks post-surgery- this would make measuring or manipulating proinflammatory mediators in the specific region problematic when the baseline has already been disrupted (Holguin et al., 2007).

The acute hyperthermia that opiates such as heroin are have been shown in the literature to be μ receptor mediated, and is likely to be caused directly by μ receptors on temperature sensitive and insensitive neurons in the hypothalamus. Short of showing directly that μ receptors are activated in the hypothalamus during hyperthermia, *in vivo* work is limited at this point in time until this is possible to do in a live organism. However, the technology to determine μ receptor activation is possible for *in vitro* work, and this could be a potential avenue of research to further the determination of heroin-induced hyperthermia.

The latter two chapters (Chapters 3 and 4) of this dissertation addressed the peripheral immune challenge of LPS in conjunction with heroin administration. The pathways proposed in the General Discussion section are specific for events which begin with a peripheral immune challenge, such as in the studies presented in this dissertation that utilize subcutaneously administered LPS. While these are perhaps

more clinically relevant (i.e., most infections begin in the periphery), there are other ways to induce an immune response in the brain. There are numerous studies that show that LPS or other immune challenges injected directly into the brain, either site-specifically or into the ventricles, also causes an inflammatory response in the brain (Aid, Langenbach, & Bosetti, 2008). Obviously, this type of immune activation is unlikely to begin with peripheral immune cells, or even spinal responses. Injection of LPS directly into the brain would help elucidate neural pathways and localize key regions involved in these processes, particularly if neurotransmitters could be specifically measured or manipulated.

The relationship between neurotransmitters and cytokines is one that is at best still poorly understood, and much work remains to be done in this area. Future studies that examine both cytokine and neurotransmitters in the same experiment would be of use to determine the neuroimmune pathways I have proposed in this dissertation. For instance, to determine the role of norepinephrine in the communication between the peripheral immune system and the central immune reaction, I would propose the completion of a series of studies that site-specifically antagonized norepinephrine in the brain regions proposed to be involved in the communication: NTS, LC, and hypothalamus and/or hippocampus. Systemic blockade of norepinephrine has already been shown to attenuate neuroinflammatory events in the hippocampus and cortex, but the addition of site-specific antagonism would contribute a great deal to understanding the particular brain regions involved (O'Sullivan, Ryan, Curtin, Harkin, & Connor, 2009; Szelenyi & Vizi, 2007). Since there seems to be contradictory evidence regarding the necessity of norepinephrine

in the hippocampus for sickness-related reduction in activity, it would be worthwhile to measure serotonin, through microdialysis or other molecular techniques, while site-specifically antagonizing norepinephrine to determine whether norepinephrine influences serotonin or if these actions are separate in this paradigm. Similarly, performing site-specific injections of antagonists for serotonin during an immune challenge would further elucidate the role of this neurotransmitter in the neuroimmune pathway, particularly as it relates to behavioral depression.

To further examine the mechanisms of how heroin produces immunosuppression in the brain and suppresses sickness behaviors, I would propose the use of site specific μ agonists to determine which brain regions appear to have the most control over heroin-induced immunosuppression in the brain. As listed above, there are many brain regions associated with μ receptors, including the LC and PAG regions. These regions would likely be the first to be examined in these types of studies. Since endogenous opiates may also play a role in regulating normal immune responses to LPS and other infections, it may also be of interest to use μ receptor antagonists to see if it is possible to potentiate the immune response to LPS. Likewise, although the μ receptor is the most obvious choice for heroin treatment to directly affect, κ and δ opioid receptors should also be examined for potential modulation of central immune responses. Since all of these proposed studies are aimed at attempting to delineate pathways in the neuroimmune response and potential effects of opiates, the use of knockout or transgenic animals would be unlikely to provide much benefit, unless those genetically altered animals are

conditional knockouts and/or restricted to a particular region or cell type (i.e., macrophages lacking μ receptors).

Conclusion

In conclusion, the data presented in this dissertation are exciting evidence contributing to our knowledge of neuroinflammatory events and sickness behavior, and the first to indicate that opiates – specifically, heroin- affect proinflammatory mediators in the brain. We demonstrated that heroin produces a proinflammatory mediator-independent hyperthermia. Furthermore, heroin suppressed all measured proinflammatory mediators in the hypothalamus as well as two key proinflammatory cytokines, IL-6 and TNF-α, in the hippocampus. Heroin also attenuated the sickness behaviors of fever, which is associated with the hypothalamus, and behavioral depression, which is associated with the hippocampus.

These results lay the groundwork for future studies to examine the relationships between proinflammatory mediators, specific brain regions, sickness behaviors, and opiates. The experiments in this dissertation are designed to establish the effects of opiates in general on proinflammatory mediators and sickness behaviors. These experiments have accomplished this goal, but it should be noted that the relationship between the proinflammatory mediators in the specific brain regions is correlational at best with the sickness behaviors exhibited. Now that the effects of heroin on proinflammatory mediators and sickness behavior have been demonstrated, future studies should focus on determining causation for the many factors that are still undetermined: brain region circuitry and pathways in the

neuroimmune communication, interactions between proinflammatory mediators and neurotransmitters, and potential localization and specific effects of μ agonist action. There remains much work to be done in this field but the observations reported here are the first steps in understanding the complex interactions of neuroimmune responses and associated behaviors with opiates.

References

1. Abraham, J. & Johnson, R. W. (2009). Central inhibition of interleukin-1beta ameliorates sickness behavior in aged mice. *Brain Behav.Immun., 23,* 396-401.

2. Aghajanian, G. K. & Sanders-Bush, E. (2002). Serotonin. In K.L.Davis, D. Charney, J. T. Coyle, & C. Nemeroff (Eds.), *Neuropsychopharmacology: The Fifth Generation of Progress* (Lippincott Williams & Wilkins.

3. Aicher, S. A., Goldberg, A., Sharma, S., & Pickel, V. M. (2000). mu-opioid receptors are present in vagal afferents and their dendritic targets in the medial nucleus tractus solitarius. *J.Comp Neurol., 422,* 181-190.

4. Aid, S., Langenbach, R., & Bosetti, F. (2008). Neuroinflammatory response to lipopolysaccharide is exacerbated in mice genetically deficient in cyclooxygenase-2. *J.Neuroinflammation., 5,* 17.

5. Anisman, H., Gibb, J., & Hayley, S. (2008). Influence of continuous infusion of interleukin-1beta on depression-related processes in mice: corticosterone, circulating cytokines, brain monoamines, and cytokine mRNA expression. *Psychopharmacology (Berl), 199,* 231-244.

6. Aston-Jones, G., Shipley, M. T., Chouvet, G., Ennis, M., van, B. E., Pieribone, V. et al. (1991). Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. *Prog.Brain Res., 88,* 47-75.

7. Atweh, S. F., Murrin, L. C., & Kuhar, M. J. (1978). Presynaptic localization of opiate receptors in the vagal and accessory optic systems: an autoradiographic study. *Neuropharmacology, 17,* 65-71.

8. Azmitia, E. C. & Segal, M. (1978). An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat. *J.Comp Neurol., 179,* 641-667.

9. Baker, A. K. & Meert, T. F. (2002). Functional effects of systemically administered agonists and antagonists of mu, delta, and kappa opioid receptor subtypes on body temperature in mice. *J.Pharmacol.Exp.Ther., 302,* 1253-1264.

10. Baldino, F., Jr., Beckman, A. L., & Adler, M. W. (1980). Actions of iontophoretically applied morphine on hypothalamic thermosensitive units. *Brain Res., 196,* 199-208.

11. Barak, O., Goshen, I., Ben-Hur, T., Weidenfeld, J., Taylor, A. N., & Yirmiya, R. (2002a). Involvement of brain cytokines in the neurobehavioral disturbances induced by HIV-1 glycoprotein120. *Brain Res., 933,* 98-108.

12. Barak, O., Weidenfeld, J., Goshen, I., Ben-Hur, T., Taylor, A. N., & Yirmiya, R. (2002b). Intracerebral HIV-1 glycoprotein 120 produces sickness behavior and pituitary-adrenal activation in rats: role of prostaglandins. *Brain Behav.Immun., 16,* 720-735.

13. Barkhudaryan, N. & Dunn, A. J. (1999). Molecular mechanisms of actions of interleukin-6 on the brain, with special reference to serotonin and the hypothalamo-pituitary-adrenocortical axis. *Neurochem.Res., 24,* 1169-1180.

14. Barrientos, R. M., Frank, M. G., Hein, A. M., Higgins, E. A., Watkins, L. R., Rudy, J. W. et al. (2009). Time course of hippocampal IL-1 beta and memory consolidation impairments in aging rats following peripheral infection. *Brain Behav.Immun., 23,* 46-54.

15. Barrientos, R. M., Watkins, L. R., Rudy, J. W., & Maier, S. F. (2009). Characterization of the sickness response in young and aging rats following E. coli infection. *Brain Behav.Immun.*.

16. Benamar, K., Geller, E. B., & Adler, M. W. (2002). Effect of a mu-opioid receptor-selective antagonist on interleukin-6 fever. *Life Sci., 70,* 2139-2145.

17. Benamar, K., McMenamin, M., Geller, E. B., Chung, Y. G., Pintar, J. E., & Adler, M. W. (2005). Unresponsiveness of mu-opioid receptor knockout mice to lipopolysaccharide-induced fever. *Br.J.Pharmacol., 144,* 1029-1031.

18. Benamar, K., Xin, L., Geller, E. B., & Adler, M. W. (2000). Blockade of lipopolysaccharide-induced fever by a mu-opioid receptor-selective antagonist in rats. *Eur.J.Pharmacol., 401,* 161-165.

19. Benamar, K., Yondorf, M., Barreto, V. T., Geller, E. B., & Adler, M. W. (2007). Deletion of mu-opioid receptor in mice alters the development of acute neuroinflammation. *J.Pharmacol.Exp.Ther., 323,* 990-994.

20. Bernheim, H. A. & Kluger, M. J. (1976). Fever: effect of drug-induced antipyresis on survival. *Science, 193,* 237-239.

21. Bertolucci, M., Perego, C., & De Simoni, M. G. (1997). Interleukin-6 is differently modulated by central opioid receptor subtypes. *Am.J.Physiol, 273,* R956-R959.

22. Bhargava, H. N., Rahmani, N. H., Villar, V. M., & Larsen, A. K. (1993). Effects of naltrexone on pharmacodynamics and pharmacokinetics of intravenously administered morphine in the rat. *Pharmacology, 46,* 66-74.

23. Bland, B. H. & Vanderwolf, C. H. (1972). Diencephalic and hippocampal mechanisms of motor activity in the rat: effects of posterior hypothalamic stimulation on behavior and hippocampal slow wave activity. *Brain Res., 43,* 67-88.

24. Blandino, P., Jr., Barnum, C. J., & Deak, T. (2006). The involvement of norepinephrine and microglia in hypothalamic and splenic IL-1beta responses to stress. *J.Neuroimmunol., 173,* 87-95.

25. Blatteis, C. M., Li, S., Li, Z., Feleder, C., & Perlik, V. (2005). Cytokines, PGE2 and endotoxic fever: a re-assessment. *Prostaglandins Other Lipid Mediat., 76,* 1-18.

26. Blatteis, C. M., Sehic, E., & Li, S. (2000). Complement and the pathogenesis of endotoxic fever. *Int.J.Biometeorol., 43,* 176-183.

27. Blatteis, C. M., Xin, L., & Quan, N. (1991). Neuromodulation of fever: apparent involvement of opioids. *Brain Res.Bull., 26,* 219-223.

28. Bluthe, R. M., Michaud, B., Poli, V., & Dantzer, R. (2000). Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice. *Physiol Behav., 70,* 367-373.

29. Bonnet, M. P., Beloeil, H., Benhamou, D., Mazoit, J. X., & Asehnoune, K. (2008). The mu opioid receptor mediates morphine-induced tumor necrosis factor and interleukin-6 inhibition in toll-like receptor 2-stimulated monocytes. *Anesth.Analg., 106,* 1142-9, table.

30. Boulant, J. A. (1998). Hypothalamic neurons. Mechanisms of sensitivity to temperature. *Ann.N.Y.Acad.Sci., 856,* 108-115.

31. Campisi, J., Hansen, M. K., O'Connor, K. A., Biedenkapp, J. C., Watkins, L. R., Maier, S. F. et al. (2003). Circulating cytokines and endotoxin are not necessary for the activation of the sickness or corticosterone response produced by peripheral E. coli challenge. *J.Appl.Physiol, 95,* 1873-1882.

32. Cannon, J. G. (2000). Inflammatory Cytokines in Nonpathological States. *News Physiol Sci., 15,* 298-303.

33. Carlson, S. L., Felten, D. L., Livnat, S., & Felten, S. Y. (1987). Alterations of monoamines in specific central autonomic nuclei following immunization in mice. *Brain Behav.Immun., 1,* 52-63.

34. Carr, D. J., Gebhardt, B. M., & Paul, D. (1993). Alpha adrenergic and mu-2 opioid receptors are involved in morphine-induced suppression of splenocyte natural killer activity. *J.Pharmacol.Exp.Ther., 264,* 1179-1186.

35. Cartmell, T., Luheshi, G. N., & Rothwell, N. J. (1999). Brain sites of action of endogenous interleukin-1 in the febrile response to localized inflammation in the rat. *J.Physiol, 518 (Pt 2),* 585-594.

36. Chen, J., Buchanan, J. B., Sparkman, N. L., Godbout, J. P., Freund, G. G., & Johnson, R. W. (2008). Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. *Brain Behav.Immun., 22,* 301-311.

37. Chen, X. H., Geller, E. B., DeRiel, J. K., Liu-Chen, L. Y., & Adler, M. W. (1996). Antisense confirmation of mu- and kappa-opioid receptor mediation of morphine's effects on body temperature in rats. *Drug Alcohol Depend., 43,* 119-124.

38. Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal.Biochem., 162,* 156-159.

39. Christie, M. J. (1991). Mechanisms of opioid actions on neurons of the locus coeruleus. *Prog.Brain Res., 88,* 197-205.

40. Chuang, R. Y., Suzuki, S., Chuang, T. K., Miyagi, T., Chuang, L. F., & Doi, R. H. (2005). Opioids and the progression of simian AIDS. *Front Biosci., 10,* 1666-1677.

41. Chuang, T. K., Killam, K. F., Jr., Chuang, L. F., Kung, H. F., Sheng, W. S., Chao, C. C. et al. (1995). Mu opioid receptor gene expression in immune cells. *Biochem.Biophys.Res.Commun., 216,* 922-930.

42. Conti, B., Tabarean, I., Andrei, C., & Bartfai, T. (2004). Cytokines and fever. *Front Biosci., 9,* 1433-1449.

43. Coplan, J. D. & Lydiard, R. B. (1998). Brain circuits in panic disorder. *Biol.Psychiatry, 44,* 1264-1276.

44. Corsini, E., Viviani, B., Birindelli, S., Gilardi, F., Torri, A., Codeca, I. et al. (2006). Molecular mechanisms underlying mancozeb-induced inhibition of TNF-alpha production. *Toxicol.Appl.Pharmacol., 212,* 89-98.

45. Cunningham, A. J., Murray, C. A., O'Neill, L. A., Lynch, M. A., & O'Connor, J. J. (1996). Interleukin-1 beta (IL-1 beta) and tumour necrosis factor (TNF) inhibit long-term potentiation in the rat dentate gyrus in vitro. *Neurosci.Lett., 203,* 17-20.

46. Dafters, R. & Taggart, P. (1992). Biotelemetric investigation of morphine's thermic and kinetic effects in rats. *Psychopharmacology (Berl), 106,* 195-201.

47. Dantzer, R. & Kelley, K. W. (1989). Stress and immunity: an integrated view of relationships between the brain and the immune system. *Life Sci., 44,* 1995-2008.

48. Dantzer, R. & Kelley, K. W. (2007). Twenty years of research on cytokine-induced sickness behavior. *Brain Behav.Immun., 21,* 153-160.

49. Dinarello, C. A. (2004). Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. *J.Endotoxin.Res., 10,* 201-222.

50. Dunn, A. J. (2006). Effects of cytokines and infections on brain neurochemistry. *Clin.Neurosci.Res., 6,* 52-68.

51. Dunn, A. J., Wang, J., & Ando, T. (1999). Effects of cytokines on cerebral neurotransmission. Comparison with the effects of stress. *Adv.Exp.Med.Biol., 461,* 117-127.

52. Fernandez-Alonso, A., Benamar, K., Sancibrian, M., Lopez-Valpuesta, F. J., & Minano, F. J. (1996). Role of interleukin-1 beta, interleukin-6 and macrophage inflammatory protein-1 beta in prostaglandin-E2-induced hyperthermia in rats. *Life Sci., 59,* L185-L190.

53. Geller, E. B., Hawk, C., Keinath, S. H., Tallarida, R. J., & Adler, M. W. (1983). Subclasses of opioids based on body temperature change in rats: acute subcutaneous administration. *J.Pharmacol.Exp.Ther., 225,* 391-398.

54. Geller, E. B., Hawk, C., Tallarida, R. J., & Adler, M. W. (1982). Postulated thermoregulatory roles for different opiate receptors in rats. *Life Sci., 31,* 2241-2244.

55. Griffin, J. D. (1999). Temperature regulation. In G.Adelman & B. H. Smith (Eds.), *Elsevier's Encyclopedia of Neuroscience* (2nd enlarged and revised edition ed., pp. 2020-2022). Elsevier Science.

56. Halloran, L. L. & Bernard, D. W. (2004). Management of drug-induced hyperthermia. *Curr.Opin.Pediatr., 16,* 211-215.

57. Handler, C. M., Geller, E. B., & Adler, M. W. (1992). Effect of mu-, kappa-, and delta-selective opioid agonists on thermoregulation in the rat. *Pharmacol.Biochem.Behav., 43,* 1209-1216.

58. Hansen, M. K., Nguyen, K. T., Goehler, L. E., Gaykema, R. P., Fleshner, M., Maier, S. F. et al. (2000). Effects of vagotomy on lipopolysaccharide-induced brain interleukin-1beta protein in rats. *Auton.Neurosci., 85,* 119-126.

59. Harden, L. M., du, P., I, Poole, S., & Laburn, H. P. (2008). Interleukin (IL)-6 and IL-1 beta act synergistically within the brain to induce sickness behavior and fever in rats. *Brain Behav.Immun., 22,* 838-849.

60. Harden, L. M., du, P., I, Poole, S., & Laburn, H. P. (2006). Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behavior. *Physiol Behav., 89,* 146-155.

61. Hart, B. L. (1988b). Biological basis of the behavior of sick animals. *Neurosci.Biobehav.Rev., 12,* 123-137.

62. Hart, B. L. (1988a). Biological basis of the behavior of sick animals. *Neurosci.Biobehav.Rev., 12,* 123-137.

63. Hauss-Wegrzyniak, B., Lynch, M. A., Vraniak, P. D., & Wenk, G. L. (2002). Chronic brain inflammation results in cell loss in the entorhinal cortex and impaired LTP in perforant path-granule cell synapses. *Exp.Neurol., 176,* 336-341.

64. Hein, A. M., Stutzman, D. L., Bland, S. T., Barrientos, R. M., Watkins, L. R., Rudy, J. W. et al. (2007). Prostaglandins are necessary and sufficient to induce contextual fear learning impairments after interleukin-1 beta injections into the dorsal hippocampus. *Neuroscience, 150,* 754-763.

65. Henry, C. J., Huang, Y., Wynne, A., Hanke, M., Himler, J., Bailey, M. T. et al. (2008). Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. *J.Neuroinflammation., 5,* 15.

66. Holguin, A., Frank, M. G., Biedenkapp, J. C., Nelson, K., Lippert, D., Watkins, L. R. et al. (2007). Characterization of the temporo-spatial effects of chronic bilateral intrahippocampal cannulae on interleukin-1beta. *J.Neurosci.Methods, 161,* 265-272.

67. Hopkins, S. J. (2007). Central nervous system recognition of peripheral inflammation: a neural, hormonal collaboration. *Acta Biomed., 78 Suppl 1,* 231-247.

68. Hori, T., Nakashima, T., Take, S., Kaizuka, Y., Mori, T., & Katafuchi, T. (1991). Immune cytokines and regulation of body temperature, food intake and cellular immunity. *Brain Res.Bull., 27,* 309-313.

69. Iwasaki, A. & Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. *Science, 327,* 291-295.

70. Jacobs, B. L. & Fornal, C. A. (1995). Serotonin and Behavior: A General Hypothesis. In F.E.Bloom & D. J. Kupfer (Eds.), *Psychopharmacology: the Fourth Generation of Progress* (4th ed., Lippincott Williams & Wilkins; 4th edition.

71. Jansky, L., Vybiral, S., Pospisilova, D., Roth, J., Dornand, J., Zeisberger, E. et al. (1995). Production of systemic and hypothalamic cytokines during the early phase of endotoxin fever. *Neuroendocrinology, 62,* 55-61.

72. Jezova, D., Vigas, M., Tatar, P., Jurcovicova, J., & Palat, M. (1985). Rise in plasma beta-endorphin and ACTH in response to hyperthermia in sauna. *Horm.Metab Res., 17,* 693-694.

73. Jolas, T. & Aghajanian, G. K. (1997). Opioids suppress spontaneous and NMDA-induced inhibitory postsynaptic currents in the dorsal raphe nucleus of the rat in vitro. *Brain Res., 755,* 229-245.

74. Kiyatkin, E. A. & Wise, R. A. (2002). Brain and body hyperthermia associated with heroin self-administration in rats. *J.Neurosci., 22,* 1072-1080.

75. Kluger, M. J. (1978). The evolution and adaptive value of fever. *Am.Sci., 66,* 38-43.

76. Kluger, M. J., Kozak, W., Conn, C. A., Leon, L. R., & Soszynski, D. (1996). The adaptive value of fever. *Infect.Dis.Clin.North Am., 10,* 1-20.

77. Kluger, M. J., Kozak, W., Conn, C. A., Leon, L. R., & Soszynski, D. (1998). Role of fever in disease. *Ann.N.Y.Acad.Sci., 856,* 224-233.

78. Kluger, M. J., Kozak, W., Leon, L. R., Soszynski, D., & Conn, C. A. (1995). Cytokines and fever. *Neuroimmunomodulation., 2,* 216-223.

79. Kluger, M. J., Ringler, D. H., & Anver, M. R. (1975). Fever and survival. *Science, 188,* 166-168.

80. Konsman, J. P., Parnet, P., & Dantzer, R. (2002). Cytokine-induced sickness behaviour: mechanisms and implications. *Trends Neurosci., 25,* 154-159.

81. Konsman, J. P., Veeneman, J., Combe, C., Poole, S., Luheshi, G. N., & Dantzer, R. (2008). Central nervous action of interleukin-1 mediates activation of limbic structures and behavioural depression in response to

peripheral administration of bacterial lipopolysaccharide. *Eur.J.Neurosci., 28,* 2499-2510.

82. Kozak, W., Kluger, M. J., Soszynski, D., Conn, C. A., Rudolph, K., Leon, L. R. et al. (1998). IL-6 and IL-1 beta in fever. Studies using cytokine-deficient (knockout) mice. *Ann.N.Y.Acad.Sci., 856,* 33-47.

83. Kozak, W., Zheng, H., Conn, C. A., Soszynski, D., van der Ploeg, L. H., & Kluger, M. J. (1995). Thermal and behavioral effects of lipopolysaccharide and influenza in interleukin-1 beta-deficient mice. *Am.J.Physiol, 269,* R969-R977.

84. Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci., 19,* 312-318.

85. Kubera, M., Maes, M., Kenis, G., Kim, Y. K., & Lason, W. (2005). Effects of serotonin and serotonergic agonists and antagonists on the production of tumor necrosis factor alpha and interleukin-6. *Psychiatry Res., 134,* 251-258.

86. Lacosta, S., Merali, Z., & Anisman, H. (1999). Behavioral and neurochemical consequences of lipopolysaccharide in mice: anxiogenic-like effects. *Brain Res., 818,* 291-303.

87. Lavicky, J. & Dunn, A. J. (1995). Endotoxin administration stimulates cerebral catecholamine release in freely moving rats as assessed by microdialysis. *J.Neurosci.Res., 40,* 407-413.

88. Lawson, L. J., Perry, V. H., Dri, P., & Gordon, S. (1990). Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience, 39,* 151-170.

89. Laye, S., Parnet, P., Goujon, E., & Dantzer, R. (1994). Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice. *Brain Res.Mol.Brain Res., 27,* 157-162.

90. Li, S., Goorha, S., Ballou, L. R., & Blatteis, C. M. (2003). Intracerebroventricular interleukin-6, macrophage inflammatory protein-1 beta and IL-18: pyrogenic and PGE(2)-mediated? *Brain Res., 992,* 76-84.

91. Li, S., Wang, Y., Matsumura, K., Ballou, L. R., Morham, S. G., & Blatteis, C. M. (1999). The febrile response to lipopolysaccharide is blocked in cyclooxygenase-2(-/-), but not in cyclooxygenase-1(-/-) mice. *Brain Res., 825,* 86-94.

92. Linthorst, A. C., Flachskamm, C., Holsboer, F., & Reul, J. M. (1994). Local administration of recombinant human interleukin-1 beta in the rat hippocampus increases serotonergic neurotransmission, hypothalamic-pituitary-adrenocortical axis activity, and body temperature. *Endocrinology, 135,* 520-532.

93. Linthorst, A. C. & Reul, J. M. (1998). Brain neurotransmission during peripheral inflammation. *Ann.N.Y.Acad.Sci., 840,* 139-152.

94. Mackowiak, P. A. (1998). Concepts of fever. *Arch.Intern.Med., 158,* 1870-1881.

95. Maier, S. F., Goehler, L. E., Fleshner, M., & Watkins, L. R. (1998a). The role of the vagus nerve in cytokine-to-brain communication. *Ann.N.Y.Acad.Sci., 840,* 289-300.

96. Maier, S. F. & Watkins, L. R. (1998b). Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychol.Rev., 105,* 83-107.

97. Mansour, A., Fox, C. A., Burke, S., Akil, H., & Watson, S. J. (1995). Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. *J.Chem.Neuroanat., 8,* 283-305.

98. Mansour, A., Fox, C. A., Thompson, R. C., Akil, H., & Watson, S. J. (1994). mu-Opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. *Brain Res., 643,* 245-265.

99. Manzella, J. P. & Roberts, N. J., Jr. (1979). Human macrophage and lymphocyte responses to mitogen stimulation after exposure to influenza virus, ascorbic acid, and hyperthermia. *J.Immunol., 123,* 1940-1944.

100. Martin, G. E. & Bacino, C. B. (1979). Action of intracerebrally injected beta-endorphin on the rat's core temperature. *Eur.J.Pharmacol., 59,* 227-236.

101. Mayfield, K. P., Kozak, A., Rudolph, K., & Kluger, M. J. (1998). Morphine suppresses development of fever to lipopolysaccharide in rats. *Ann.N.Y.Acad.Sci., 856,* 281-285.

102. Mellon, R. D. & Bayer, B. M. (1998). Role of central opioid receptor subtypes in morphine-induced alterations in peripheral lymphocyte activity. *Brain Res., 789,* 56-67.

103. Milligan, E. D., McGorry, M. M., Fleshner, M., Gaykema, R. P., Goehler, L. E., Watkins, L. R. et al. (1997). Subdiaphragmatic vagotomy does not prevent fever following intracerebroventricular prostaglandin E2: further

evidence for the importance of vagal afferents in immune-to-brain communication. *Brain Res., 766,* 240-243.

104. Mingam, R., Moranis, A., Bluthe, R. M., De Smedt-Peyrusse, V., Kelley, K. W., Guesnet, P. et al. (2008). Uncoupling of interleukin-6 from its signalling pathway by dietary n-3-polyunsaturated fatty acid deprivation alters sickness behaviour in mice. *Eur.J.Neurosci., 28,* 1877-1886.

105. Mocchetti, I., Ritter, A., & Costa, E. (1989). Down-regulation of proopiomelanocortin synthesis and beta-endorphin utilization in hypothalamus of morphine-tolerant rats. *J.Mol.Neurosci., 1,* 33-38.

106. Myers, R. D., Lopez-Valpuesta, F. J., Minano, F. J., Wooten, M. H., Barwick, V. S., & Wolpe, S. D. (1994). Fever and feeding in the rat: actions of intrahypothalamic interleukin-6 compared to macrophage inflammatory protein-1 beta (MIP-1 beta). *J.Neurosci.Res., 39,* 31-37.

107. Nakajima, K., Matsushita, Y., Tohyama, Y., Kohsaka, S., & Kurihara, T. (2006). Differential suppression of endotoxin-inducible inflammatory cytokines by nuclear factor kappa B (NFkappaB) inhibitor in rat microglia. *Neurosci.Lett., 401,* 199-202.

108. Nakashima, T., Hori, T., Mori, T., Kuriyama, K., & Mizuno, K. (1989). Recombinant human interleukin-1 beta alters the activity of preoptic thermosensitive neurons in vitro. *Brain Res.Bull., 23,* 209-213.

109. Newman, E. J., Grosset, D. G., & Kennedy, P. G. (2009). The parkinsonism-hyperpyrexia syndrome. *Neurocrit.Care, 10,* 136-140.

110. Nilsberth, C., Elander, L., Hamzic, N., Norell, M., Lonn, J., Engstrom, L. et al. (2009a). The role of interleukin-6 in lipopolysaccharide-induced fever by mechanisms independent of prostaglandin E2. *Endocrinology, 150,* 1850-1860.

111. Nilsberth, C., Hamzic, N., Norell, M., & Blomqvist, A. (2009b). Peripheral lipopolysaccharide administration induces cytokine mRNA expression in the viscera and brain of fever-refractory mice lacking microsomal prostaglandin E synthase-1. *J.Neuroendocrinol., 21,* 715-721.

112. O'Connor, K. A., Johnson, J. D., Hansen, M. K., Wieseler Frank, J. L., Maksimova, E., Watkins, L. R. et al. (2003). Peripheral and central proinflammatory cytokine response to a severe acute stressor. *Brain Res., 991,* 123-132.

113. O'Neill, L. A. & Kaltschmidt, C. (1997). NF-kappa B: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci., 20,* 252-258.

114. O'Sullivan, J. B., Ryan, K. M., Curtin, N. M., Harkin, A., & Connor, T. J. (2009). Noradrenaline reuptake inhibitors limit neuroinflammation in rat cortex following a systemic inflammatory challenge: implications for depression and neurodegeneration. *Int.J.Neuropsychopharmacol., 12,* 687-699.

115. Pacifici, R., Di, C. S., Bacosi, A., Pichini, S., & Zuccaro, P. (2000). Pharmacokinetics and cytokine production in heroin and morphine-treated mice. *Int.J.Immunopharmacol., 22,* 603-614.

116. Pauli, S., Linthorst, A. C., & Reul, J. M. (1998). Tumour necrosis factor-alpha and interleukin-2 differentially affect hippocampal serotonergic neurotransmission, behavioural activity, body temperature and hypothalamic-pituitary-adrenocortical axis activity in the rat. *Eur.J.Neurosci., 10,* 868-878.

117. Paxinos, G. & Watson, C. (2005). *The rat brain in stereotaxic coordinates*. (Fifth Edition ed.) New York, NY: Elsevier.

118. Perry, V. H., Hume, D. A., & Gordon, S. (1985). Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience, 15,* 313-326.

119. Poole, S. L., Deuchars, J., Lewis, D. I., & Deuchars, S. A. (2007). Subdivision-specific responses of neurons in the nucleus of the tractus solitarius to activation of mu-opioid receptors in the rat. *J.Neurophysiol., 98,* 3060-3071.

120. Prakash, U. & Dey, P. K. (1981). Morphine hyperthermia in rats: role of neurochemical substances in brain. *Indian J.Physiol Pharmacol., 25,* 237-245.

121. Pugh, C. R., Kumagawa, K., Fleshner, M., Watkins, L. R., Maier, S. F., & Rudy, J. W. (1998). Selective effects of peripheral lipopolysaccharide administration on contextual and auditory-cue fear conditioning. *Brain Behav.Immun., 12,* 212-229.

122. Rachal, P. C., Fleshner, M., Watkins, L. R., Maier, S. F., & Rudy, J. W. (2001). The immune system and memory consolidation: a role for the cytokine IL-1beta. *Neurosci.Biobehav.Rev., 25,* 29-41.

123. Rezvani, A. H., Gordon, C. J., & Heath, J. E. (1982). Action of preoptic injections of beta-endorphin on temperature regulation in rabbits. *Am.J.Physiol, 243,* R104-R111.

124. Rhim, H. & Miller, R. J. (1994). Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius of the rat. *J.Neurosci., 14,* 7608-7615.

125. Richwine, A. F., Sparkman, N. L., Dilger, R. N., Buchanan, J. B., & Johnson, R. W. (2009). Cognitive deficits in interleukin-10-deficient mice after peripheral injection of lipopolysaccharide. *Brain Behav.Immun., 23,* 794-802.

126. Rusyniak, D. E. & Sprague, J. E. (2005). Toxin-induced hyperthermic syndromes. *Med.Clin.North Am., 89,* 1277-1296.

127. Scammell, T. E., Griffin, J. D., Elmquist, J. K., & Saper, C. B. (1998). Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever. *Am.J.Physiol, 274,* R783-R789.

128. Sebag, J., Reed, W. P., & Williams, R. C., Jr. (1977). Effect of temperature on bacterial killing by serum and by polymorphonuclear leukocytes. *Infect.Immun., 16,* 947-954.

129. Semmler, A., Okulla, T., Sastre, M., Dumitrescu-Ozimek, L., & Heneka, M. T. (2005). Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. *J.Chem.Neuroanat., 30,* 144-157.

130. Sheng, W. S., Hu, S., Ni, H. T., Rowen, T. N., Lokensgard, J. R., & Peterson, P. K. (2005). TNF-alpha-induced chemokine production and apoptosis in human neural precursor cells. *J.Leukoc.Biol., 78,* 1233-1241.

131. Sparkman, N. L., Buchanan, J. B., Heyen, J. R., Chen, J., Beverly, J. L., & Johnson, R. W. (2006). Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. *J.Neurosci., 26,* 10709-10716.

132. Staubli, U. & Otaky, N. (1994). Serotonin controls the magnitude of LTP induced by theta bursts via an action on NMDA-receptor-mediated responses. *Brain Res., 643,* 10-16.

133. Stefferl, A., Hopkins, S. J., Rothwell, N. J., & Luheshi, G. N. (1996). The role of TNF-alpha in fever: opposing actions of human and murine TNF-alpha and interactions with IL-beta in the rat. *Br.J.Pharmacol., 118,* 1919-1924.

134. Steiner, A. A., Rudaya, A. Y., Robbins, J. R., Dragic, A. S., Langenbach, R., & Romanovsky, A. A. (2005). Expanding the febrigenic role of cyclooxygenase-2 to the previously overlooked responses. *Am.J.Physiol Regul.Integr.Comp Physiol, 289,* R1253-R1257.

135. Stitt, J. T. (1979). Fever versus hyperthermia. *Fed.Proc., 38,* 39-43.

136. Szelenyi, J. & Vizi, E. S. (2007). The catecholamine cytokine balance: interaction between the brain and the immune system. *Ann.N.Y.Acad.Sci., 1113,* 311-324.

137. Tanaka, S., Ide, M., Shibutani, T., Ohtaki, H., Numazawa, S., Shioda, S. et al. (2006). Lipopolysaccharide-induced microglial activation induces learning and memory deficits without neuronal cell death in rats. *J.Neurosci.Res., 83,* 557-566.

138. Thompson, W. L., Karpus, W. J., & Van Eldik, L. J. (2008). MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. *J.Neuroinflammation., 5,* 35.

139. Thornhill, J. A., Hirst, M., & Gowdey, C. W. (1978). Changes in the hyperthermic responses of rats to daily injections of morphine and the antagonism of the acute response by naloxone. *Can.J.Physiol Pharmacol., 56,* 483-489.

140. Torrecilla, M., Marker, C. L., Cintora, S. C., Stoffel, M., Williams, J. T., & Wickman, K. (2002). G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons. *J.Neurosci., 22,* 4328-4334.

141. Tsai, S. M., Lin, M. T., Wang, J. J., & Huang, W. T. (2003). Pyrogens enhance beta-endorphin release in hypothalamus and trigger fever that can be attenuated by buprenorphine. *J.Pharmacol.Sci., 93,* 155-162.

142. Tseng, L. F. & Li, C. H. (1980). beta-Endorphin: hyperthermia in mice by intravenous injection. *Int.J.Pept.Protein Res., 15,* 471-474.

143. Van Bockstaele, E. J., Peoples, J., & Telegan, P. (1999). Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: evidence for a monosynaptic pathway. *J.Comp Neurol., 412,* 410-428.

144. Vanderwolf, C. H. (1969). Hippocampal electrical activity and voluntary movement in the rat. *Electroencephalogr.Clin.Neurophysiol., 26,* 407-418.

145. Vanderwolf, C. H. (2001). The hippocampus as an olfacto-motor mechanism: were the classical anatomists right after all? *Behav.Brain Res., 127,* 25-47.

146. Vanderwolf, C. H., McLauchlin, M., Dringenberg, H. C., & Baker, G. B. (1997). Brain structures involved in the behavioral stimulant effect of central serotonin release. *Brain Res., 772,* 121-134.

147. Vescovi, P. P. & Coiro, V. (1993). Hyperthermia and endorphins. *Biomed.Pharmacother., 47,* 301-304.

148. Vescovi, P. P., Coiro, V., Volpi, R., Giannini, A., & Passeri, M. (1992). Hyperthermia in sauna is unable to increase the plasma levels of ACTH/cortisol, beta-endorphin and prolactin in cocaine addicts. *J.Endocrinol.Invest, 15,* 671-675.

149. Vescovi, P. P., Pedrazzoni, M., Gerra, G., Pioli, G., Maninetti, L., Michelini, M. et al. (1989). Impaired ACTH and beta-endorphin response to sauna-induced hyperthermia in heroin addicts. *Acta Endocrinol.(Copenh), 121,* 484-488.

150. Vitkovic, L., Konsman, J. P., Bockaert, J., Dantzer, R., Homburger, V., & Jacque, C. (2000). Cytokine signals propagate through the brain. *Mol.Psychiatry, 5,* 604-615.

151. Wallenstein, M. C. (1983). Effect of prostaglandin synthetase inhibitors on non-analgesic actions of morphine. *Eur.J.Pharmacol., 90,* 65-73.

152. Wang, J., Barke, R. A., Ma, J., Charboneau, R., & Roy, S. (2008). Opiate abuse, innate immunity, and bacterial infectious diseases. *Arch.Immunol.Ther.Exp.(Warsz.), 56,* 299-309.

153. Wang, J. & Dunn, A. J. (1998). Mouse interleukin-6 stimulates the HPA axis and increases brain tryptophan and serotonin metabolism. *Neurochem.Int., 33,* 143-154.

154. Watkins, L. R., Goehler, L. E., Relton, J. K., Tartaglia, N., Silbert, L., Martin, D. et al. (1995). Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. *Neurosci.Lett., 183,* 27-31.

155. Watkins, L. R., Maier, S. F., & Goehler, L. E. (1995). Cytokine-to-brain communication: a review & analysis of alternative mechanisms. *Life Sci., 57,* 1011-1026.

156. Wolff, S., Klatt, S., Wolff, J. C., Wilhelm, J., Fink, L., Kaps, M. et al. (2009). Endotoxin-induced gene expression differences in the brain and effects of iNOS inhibition and norepinephrine. *Intensive Care Med., 35,* 730-739.

157. Yakimova, K. S., Sann, H., & Pierau, F. K. (1998). Effects of kappa and delta opioid agonists on activity and thermosensitivity of rat hypothalamic neurons. *Brain Res., 786,* 133-142.

158. Yakimova, K. S., Sann, H., & Pierau, F. K. (1996). Neuronal basis for the hyperthermic effect of mu-opioid agonists in rats: decrease in temperature sensitivity of warm-sensitive hypothalamic neurons. *Neurosci.Lett., 218,* 115-118.

159. Yoo, J. H., Yang, E. M., Lee, S. Y., Loh, H. H., Ho, I. K., & Jang, C. G. (2003). Differential effects of morphine and cocaine on locomotor activity and sensitization in mu-opioid receptor knockout mice. *Neurosci.Lett., 344,* 37-40.

160. Zalcman, S., Green-Johnson, J. M., Murray, L., Nance, D. M., Dyck, D., Anisman, H. et al. (1994). Cytokine-specific central monoamine alterations induced by interleukin-1, -2 and -6. *Brain Res., 643,* 40-49.

161. Zhao, X., Zhang, Y., Strong, R., Zhang, J., Grotta, J. C., & Aronowski, J. (2007). Distinct patterns of intracerebral hemorrhage-induced alterations in NF-kappaB subunit, iNOS, and COX-2 expression. *J.Neurochem., 101,* 652-663.